The Institute of Paper Chemistry

Appleton, Wisconsin

Doctor's Dissertation

The Methanol-Extractable Aromatic Materials in the Inner Bark of *P. Tremuloides*

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June, 1959

THE METHANOL-EXTRACTABLE AROMATIC MATERIALS IN THE INNER BARK OF *P. tremuloides*

A thesis submitted by

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GLOSSARY

1. Definition of Terms Used:

   (a) Inner phloem -- that portion of phloem tissue which lies between the cambial zone and the last-formed stone cells.

   (b) Stone cell layer -- that portion of phloem tissue which lies between the inner phloem and periderm layer.

   (c) Whole inner bark -- a combination of inner phloen and stone cell layer.

   (d) Whole bark -- all tissue lying outside the cambial layer.

   (e) Soft xylem -- newly formed tissue containing 5-10% lignin.

   (f) T-74,75 -- a composite sample from tree T-74 and tree T-75.

   (g) Wiesner-positive -- giving a red-violet color when treated with the Wiesner reagent (1% phloroglucinol in 12% hydrochloric acid solution).

   (h) Mäule-positive -- giving a cerise color when subjected to chlorination followed by treatment with a freshly prepared solution of 10% sodium sulfite.

2. Paper Chromatographic Developers:

   (a) TAW, toluene-acetic acid-water (4:1:5).

   (b) BAW, n-butanol-acetic acid-water (4:1:5).

   (c) BF, benzene saturated with 98% formic acid.

   (d) NBEW, n-butyl ether saturated with water.

   (e) BAm, n-butanol saturated with a 2% aqueous ammonia solution.

   (f) BAmC, n-butanol saturated with an aqueous solution of 1.5N ammonia and 1.5N ammonium carbonate.

(g) BzAW, benzene-acetic acid-water (2:2:1).

(h) BEAmC, n-butanol-ethanol-1.5N ammonia, 1.5N ammonium carbonate (40:11:19).

(i) BPW, n-butanol-pyridine-water (10:3:3).

(j) PFW, phenol-formic acid-water (3:0.01:1).

3. Qualitative Indicator Reagents for Materials on Paper Chromatograms:

(a) Wiesner reagent, 1% phloroglucinol in 12% hydrochloric acid solution.

(b) Mäule test, chlorination of the damp chromatogram followed by spraying with a freshly prepared 10% sodium sulfite solution.

(c) pA, p-anisidine hydrochloride dissolved in n-butanol.

(d) DNPH, a 12% hydrochloric acid solution saturated with 2,4-dinitrophenylhydrazine.

(e) FC, a 2% absolute ethanolic solution of ferric chloride.

(f) PPN, a sodium metaperiodate solution and a piperazine-sodium nitroprusside solution used in that order to spray the chromatograms.

(g) PNA, diazotized p-nitroaniline solution followed by an over-spray of sodium carbonate.

(h) SN, 3% silver nitrate in acetone followed by a 2% alcoholic sodium hydroxide spray and finally a wash using concentrated sodium thiosulfate.

INTRODUCTION

Despite all of the research in the field of wood chemistry, the initial definition given to lignin is still appropriate, i.e., it is the fiber-encrusting material of woody plants (1). It is known today, however, that there are at least three general types of lignins, gymnosperm lignin which contains the guaiacyl nucleus, angiosperm dicotyledonous lignin which contains both the guaiacyl and syringyl nuclei, and angiosperm monocotyledonous lignin which contains the p-hydroxyphenyl nucleus in addition to the guaiacyl and syringyl nuclei. There are also analytical variations within these three general classes of lignin depending on the species (2) and even the location of the stem of the tree under consideration (3). Thus, it is necessary to qualify the term "lignin" with its botanical origin.

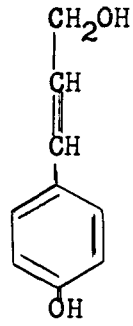
The facts concerning the structure of the lignins still do not yield a complete picture of these complex systems (4). The classical analytical approaches to this problem have proven useful, but, in this respect, studies such as degradation, extraction, and the use of model compounds have been applied for the most part to mature wood. Since the mature wood undoubtedly has been prone to many chemical changes over the years, the recent biosynthetic approaches to the formation of lignin have minimized these changes by focusing attention on the living tissues where lignification is occurring. These studies have greatly extended our concepts concerning not only the formation of the lignins but also the structure of these systems (5,6).

It is believed that the compounds which are ultimately incorporated into lignin move from the sites of synthesis, through the inner bark and to all living portions of the tree. Since the lignins are water-insoluble (1), the compounds from which they are synthesized have to be transported by the sap in a water-soluble form to the locations in the tree where they are required (7).

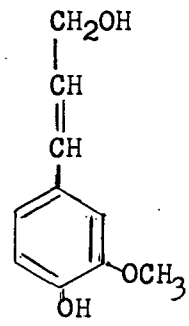
Since the functions of the inner bark include transport, storage and other biological activity, it seemed desirable to analyze this tissue for the presence of low-molecular weight precursors of lignin.

HISTORICAL REVIEW

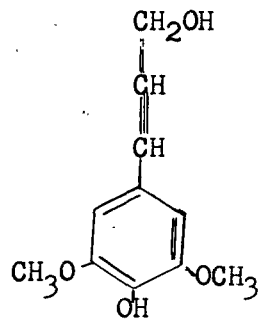
During the past decade, considerable attention has been focused on the biosynthesis of lignin. The hypothesis that coniferyl alcohol is a basic unit of gymnosperm lignin has had wide acceptance and has been fortified over the years by the weight of experimental evidence. The concept that coniferyl alcohol was associated with gymnosperm lignin was first proposed by Klason who also suggested that this highly reactive alcohol was probably bound as the glycoside coniferin until needed for lignification (8,9). Freudenberg has extended these early concepts to include the building stones for angiosperm lignins (10) (Figure 1). In trees, the glucosides of the lignin building stones, i.e., coniferin and syringin, are thought to be synthesized outside the zone of lignification and transported to the sites of lignification.



p-Coumaryl Alcohol



Coniferyl Alcohol



Sinapyl Alcohol

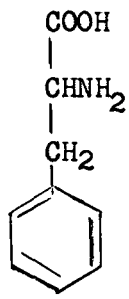
Figure 1. The Building Stones of Gymnosperm and Angiosperm Lignins

via the inner bark. Furthermore, the lignifying tissues contain enzymes which can hydrolyze these glucosides, yielding the free alcohols. Erdtman has postulated that coniferyl alcohol would react in accord with the reaction mechanism whereby isoeugenol is converted into dehydrodiisoeugenol (11). Freudenberg has extended this idea to include enzymatic coupling and polymerization of the lignin building stones (12-14).

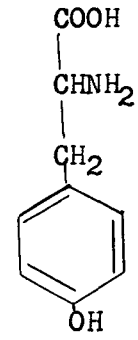
Particularly as a result of information obtained in the past decade, the newer views concerning the biochemistry of lignin have resulted in a general concept which traces the biosynthesis of lignin from its origin, via glucose, to its final deposition in the cell wall (5). This recent evidence can be divided into four groups, (1) the biosynthesis of the C_9 phenolic building stones, (2) the enzymatic studies relating to the polymerization of the lignin building stones, (3) the introduction of radioactive carbon into the living plant, and (4) analytical studies.

THE BIOSYNTHESIS OF THE LIGNIN BUILDING STONES

Since there is evidence that the aromatic ring systems of the basic building stones and of the closely related aromatic amino acids, Figure 2, originate from glucose, the question of aromatic biosynthesis is of primary interest. The pathways proposed to explain the biosynthesis of the lignin building stones can be divided into three stages, (1) the synthesis of glucose, (2) the conversion of glucose into the hydroaromatic ring system, and (3) the formation of the phenolic building stones from the hydroaromatic skeleton.



Phenylalanine



Tyrosine

Figure 2. The Common Aromatic Amino Acids

THE SYNTHESIS OF GLUCOSE

It is known that atmospheric carbon dioxide and water are brought together in the leaves where photosynthesis occurs, the water being transported from the soil via the roots and living xylem tissue. The first-formed products contain three carbon atoms and are subsequently converted into glucose and other hexoses (15,16).

THE CONVERSION OF GLUCOSE TO THE HYDROAROMATIC RING SYSTEM

The schemes presented in Figures 3 and 4 outline the biosynthesis of the hydroaromatic acids from glucose. These acids are considered to be precursors to the aromatic ring system. Most of the intermediates in these schemes have been isolated from bacterial and fungal cultures (17-31). However, since shikimic acid seems to be widespread in higher plants (32-35), it is possible that these pathways may be generally applicable to the aromatization sequence in trees.

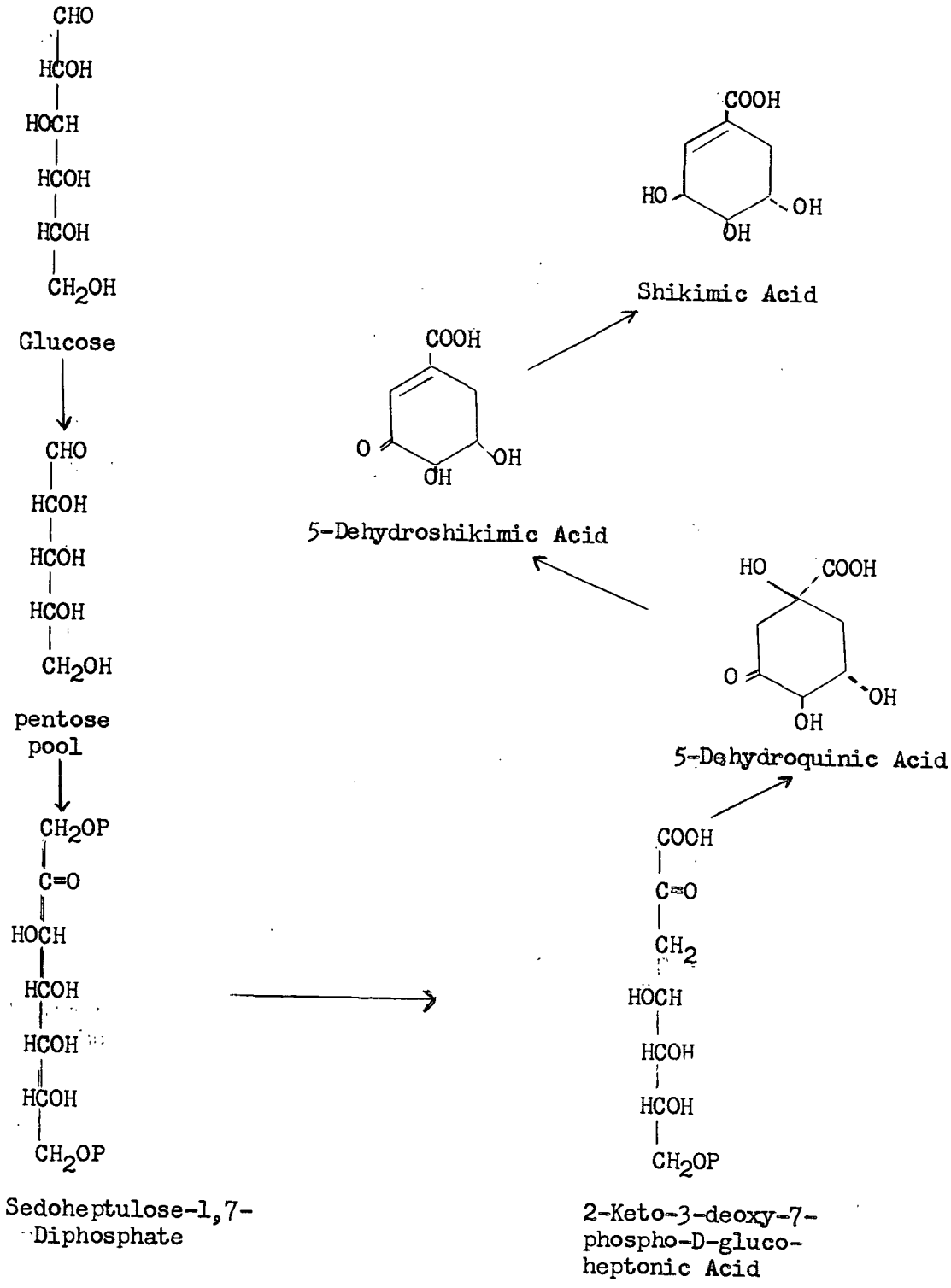


Figure 3. The Conversion of Glucose to Shikimic Acid (17-29)

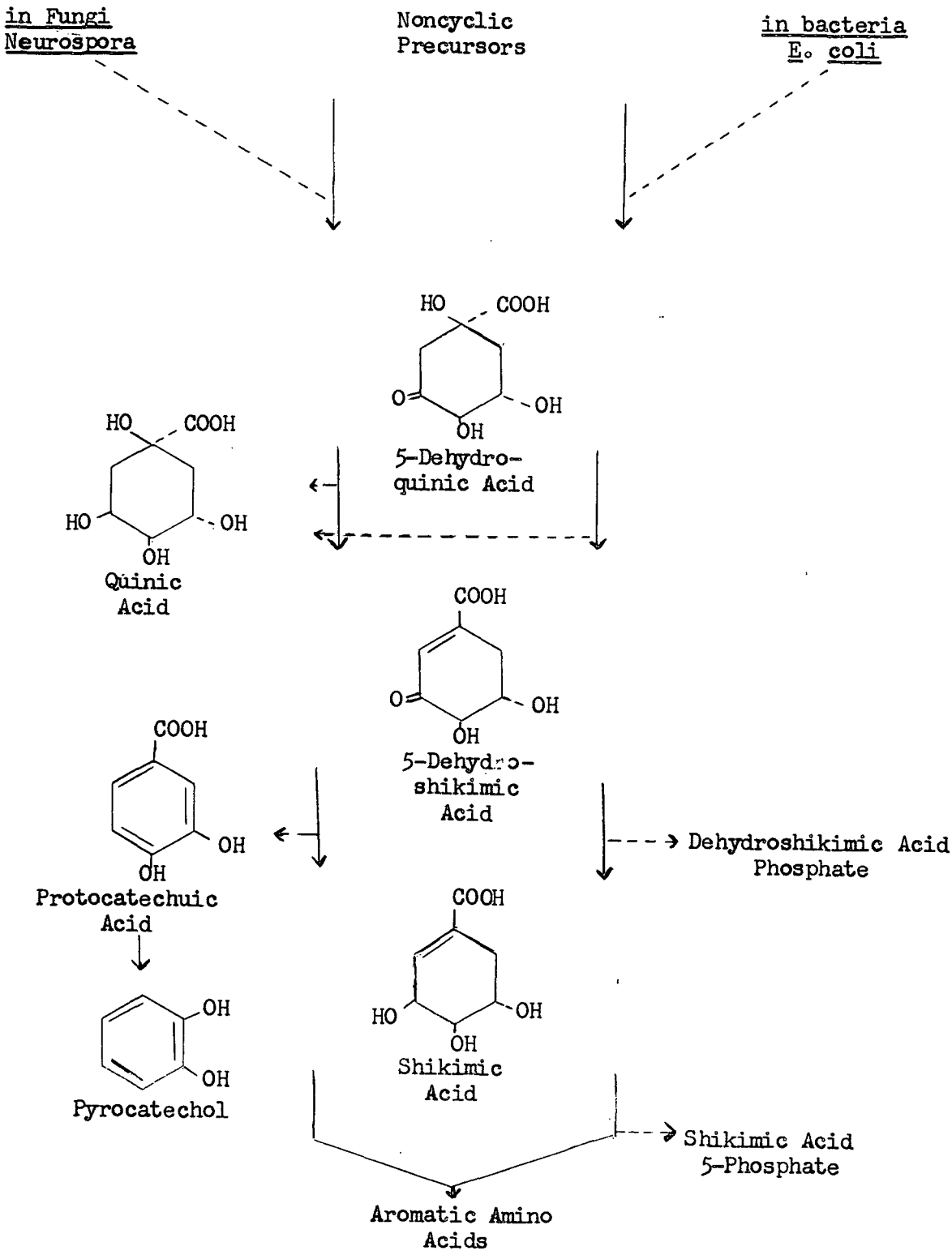


Figure 4. The Interrelationships Between the Early Alicyclic Precursors and the Aromatic Amino Acids (31)

THE FORMATION OF THE PHENOLIC BUILDING STONES

Figure 5 shows the pathway from shikimic acid to an aromatic C_6-C_3 unit (17-31). From this sequence, it appears that pyruvic acid condenses with an unknown intermediate, Z_1, yielding prephenic acid, which is structurally very similar to the aromatic C_6-C_3 lignin building stones shown in Figure 1. It should be noted that the interconversion of carbonyl groups and amino groups occurs very readily in plant metabolism and justifies the hypothesis that the aromatic amino acids may be precursors to the lignin building stones (36).

In a study concerning the biosynthesis of methyl p-methoxycinnamate by brown rot fungi, p-hydroxyphenylpyruvic acid was identified as a metabolic end product. It was suggested that this compound might also be an intermediate between shikimic acid and the lignin building stones and was therefore included in Figure 5 as a separate sequence (37-42).

The introduction of the first hydroxyl group into the aromatic nucleus has been demonstrated in animal tissue by the enzymatic conversion of phenylalanine to tyrosine (43). However, the mechanism of such hydroxylation reactions is still unknown. The enzymatic introduction of a second hydroxyl group into the aromatic ring is better understood and has been attributed to the action of the "phenolase" enzyme system, which has been isolated from mushrooms (44).

The formation of methoxyl groups in the aromatic nucleus probably consists of a direct methylation of a phenol rather than the introduction

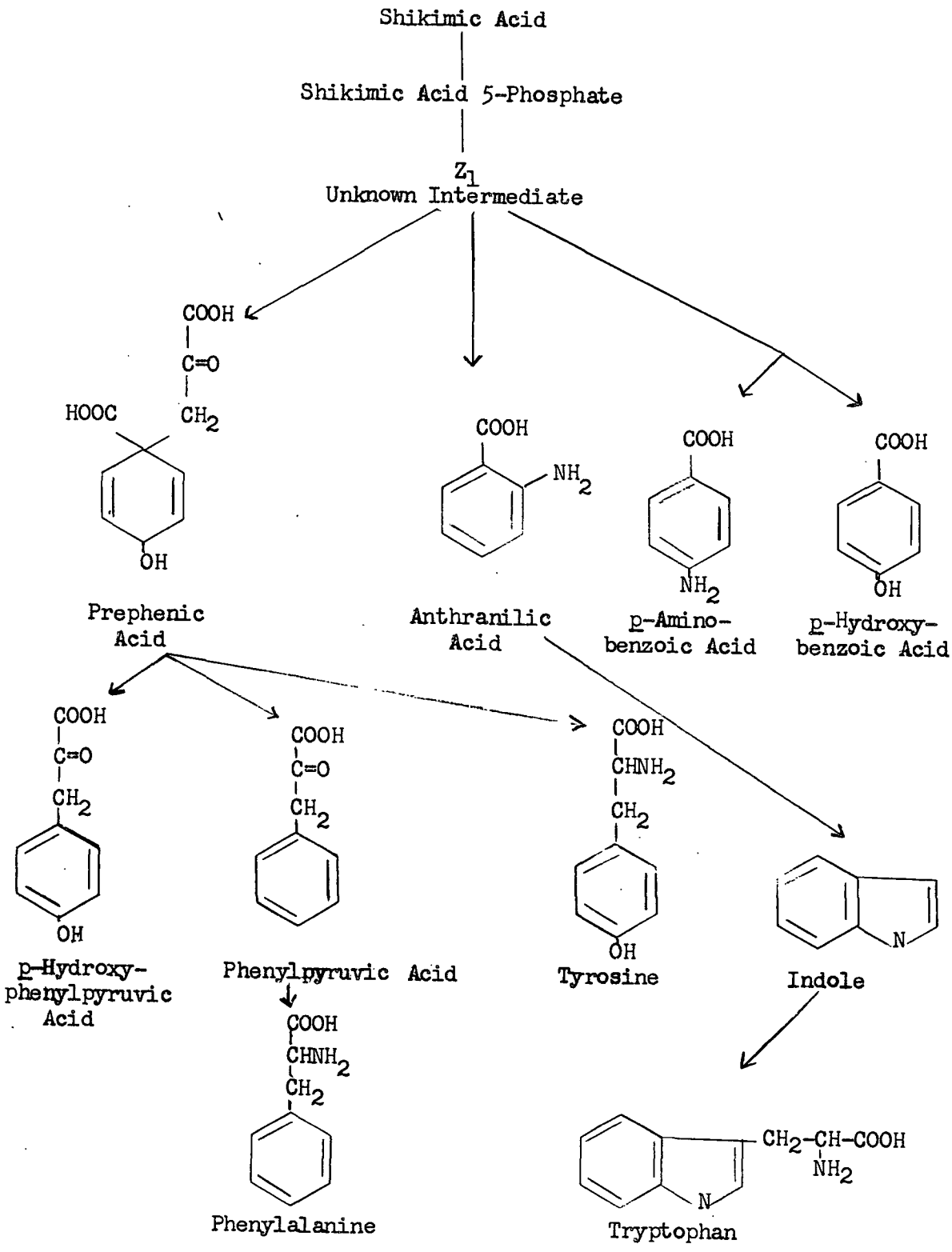


Figure 5. The Conversion of Shikimic Acid to Aromatic C_6-C_3 Compounds (17-31, 37-42)

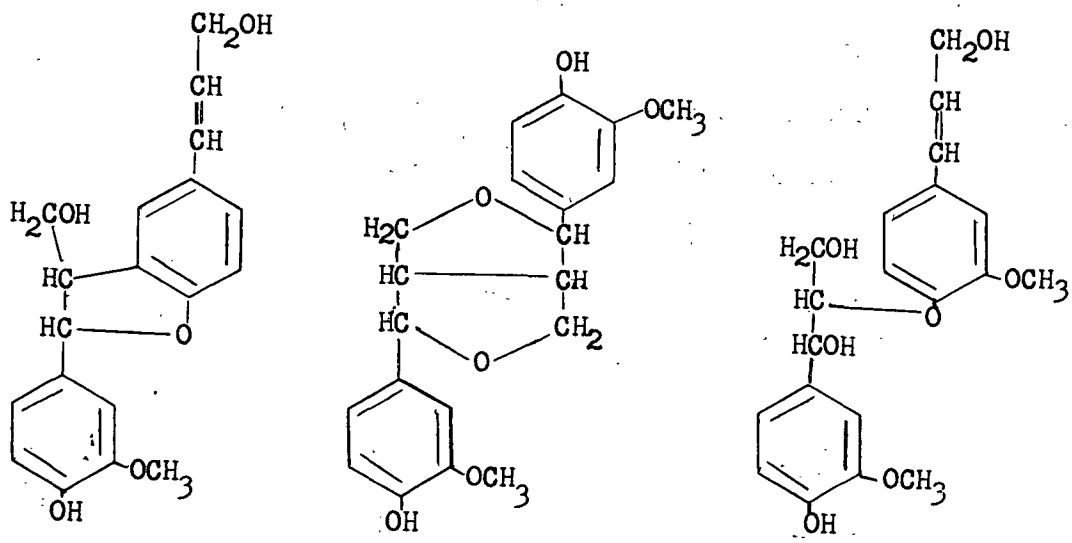
of an entire methoxyl group into the ring. Serine, glycine and methionine were capable of contributing methyl groups for this enzymatic methylation of tobacco and barley lignins (45,46).

Thus far, the evidence suggests that glucose is converted via the hydroaromatic acids into several aromatic C_6-C_3 compounds. By appropriate conversions, i.e., hydroxylation, methylation, transamination, reduction, etc., these compounds are converted into the lignin building stones (cf. Figure 8, p. 18).

ENZYMATIC CONVERSION OF THE LIGNIN BUILDING STONES

Since lignin is thought to be formed by dehydrogenative polymerization of the lignin building stones, these compounds supposedly are the substrate for nonspecific phenol-oxidizing enzymes which initiate the polymerization (5,47). It has been stated, however, that the phenol-oxidizing enzymes would probably attack any of the phenolic intermediates which are transported through the lignifying tissues or occur on the biosynthetic pathways (5). However, Freudenberg postulated that the synthesis of these building stones occurred outside the zone of lignification, and therefore some mechanism to protect the building stones while being transported to the lignifying tissues must be operative (10). Both Klason and Freudenberg have postulated that coniferyl alcohol was bound as a glucoside, coniferin, until needed for lignin formation (8,9,10). Furthermore, cell-bound enzymes capable of hydrolyzing coniferin have been discovered in the cambial region of conifers and several hardwoods (10).

In studies with isoeugenol, it was found that the dehydrogenative polymerization of this compound involved coupling not only ortho to the phenolic hydroxyl group but also at the beta-carbon atom of the side chain. It was suggested that a similar mechanism might be applicable to the polymerization of coniferyl alcohol (11). By treating coniferyl alcohol in an aqueous solution with air and a mushroom oxidase, the dimers which can be predicted by the above mechanism have been isolated (12-14) (cf. Figure 6). These dimers were then employed as starting materials with the same mushroom enzyme system; the end products of these reactions were amorphous powders similar to coniferous lignin (12,14).



Dehydrodiconiferyl Alcohol

DL-Pinoresinol

Guaiacylglycerol-beta-coniferyl Ether

Figure 6. Dimeric Products from Coniferyl Alcohol

In addition, coniferyl alcohol was reacted with this mushroom oxidase and the reaction allowed to proceed beyond the dimer stage. The resulting polymeric product was very similar to gymnosperm lignin. Finally, oxidative enzymes were isolated from the cambial sap of spruce and employed as above, yielding the same results (10,12).

These studies have been extended to the formation of hardwood lignins where it was found that sinapyl alcohol, when mixed with coniferyl alcohol and mushroom oxidase, yielded a polymeric product similar in all respects to angiosperm dicotyledonous lignin. However, sinapyl alcohol alone did not yield a ligninlike product (10,49). It was also found that the dehydrogenation polymer (DHP) of p-coumaryl alcohol was similar to the DHP from coniferyl alcohol except for the expected lack of methoxyl. In addition, the ultraviolet spectrum of the p-coumaryl alcohol DHP was similar to a lignin spectrum, but the DHP from p-coumaraldehyde yielded an ultraviolet spectrum which differed greatly from lignin (48).

It appears, therefore, that the lignin building stones are formed outside the zone of lignification. They are then combined with glucose for protection and transport from the sites of biosynthesis to the lignifying tissues. At this location, the glucosides are hydrolyzed, and, under the influence of the oxidative enzymes present, the liberated lignin building stones polymerize to yield lignin.

THE INTRODUCTION OF C^{14} INTO THE LIVING PLANT

In order to demonstrate that shikimic acid was a precursor to lignin, this acid was randomly labeled and fed to young wheat and maple plants. After one or two days of metabolism, the plant was degraded by a nitrobenzene oxidation, resulting in radioactive vanillin and syringaldehyde. It was suggested, therefore, that shikimic acid was converted into the aromatic system of lignin (50). Carrying this test one step farther, shikimic acid, specifically labeled in positions two and six, was fed to sugar cane plants. The cuoxam lignin, isolated after several days of metabolism, was oxidized with nitrobenzene. The isolated vanillin was analyzed and shown to be radioactive specifically in positions two and six. This evidence suggested that the nonaromatic skeleton of shikimic acid remained unchanged during its incorporation into lignin (51).

In further studies with wheat plants, it was concluded that aromatic C_6-C_1 compounds were not effective as lignin precursors, but aromatic C_6-C_3 compounds, such as tyrosine, phenylalanine and cinnamic acid, were quite effective. Thus, a high degree of interconvertibility among these three compounds in the metabolic pool was suggested, and by suitable modifications of the substituents, these compounds could serve as precursors to all three general types of lignin. In addition, it was shown that the radioactivity of randomly labeled ferulic acid, administered to growing wheat plants, was almost entirely recovered from the guaiacyl portion of the lignin. Thus, it was suggested that the guaiacyl

nucleus, once formed, probably did not undergo further methoxylation to yield the syringyl nucleus (52).

Labeled p-hydroxybenzaldehyde and mandelic acid were fed to wheat and maple plants. It was found that these compounds were not utilized in lignin formation, and therefore, it was concluded that the aromatic C_6-C_1 plus C_2 and the aromatic C_6-C_2 plus C_1 condensation steps were not important in the formation of the phenylpropane building stones (53).

To demonstrate the role of coniferin in gymnosperm lignification, this compound was labeled with C^{14} and fed to a young spruce tree. After two months, the radioactivity had migrated a few centimeters upward and downward from the point of injection, and more than 90% of the radioactivity was bound with the lignin (54). In addition, phenylalanine, specifically labeled at the beta-carbon atom of the side chain, was incorporated into young spruce stems. After 2-3 days of metabolism, radioactive coniferin was isolated just above the point of entry. It was concluded, therefore, that coniferin is an intermediate in lignin biosynthesis (55). This evidence also demonstrated the close relationship of the aromatic amino acid, phenylalanine, with the lignin building stone, coniferyl alcohol.

ANALYTICAL STUDIES

The concept that coniferous lignin is formed from coniferyl alcohol by enzymatic dehydrogenative polymerization must also include the uptake of water; cf. the formation of guaiacylglycerol-beta-coniferyl ether, Figure 6. Recently, Björkman obtained an isolated lignin which was

thought to be quite similar to protolignin (2, 56). The analysis of this lignin preparation is shown in Table I. The composition of the Björkman lignin compared with coniferyl alcohol revealed that the lignin had lost some hydrogen but also had acquired oxygen. It was therefore assumed that the excess oxygen originated from water, and a new calculation was made with coniferyl alcohol by adding 0.47 units of water, the amount of oxygen necessary to account for the extra oxygen in the lignin. This new calculation revealed that the lignin was deficient by 1.6 atoms of hydrogen per C_9 building unit. For pure dehydrogenation, a loss of nearly 2 hydrogen atoms would be expected. This lower value supposedly indicated that some coupling was occurring which did not involve dehydrogenation or condensation (5).

TABLE I

ANALYSIS OF BJÖRKMAN LIGNIN (5)

1. Coniferyl alcohol	C_9	H_9	O_2	$(OCH_3)_1$
2. Björkman lignin (spruce)	C_9	$H_{8.37}$	$O_{2.47}$	$(OCH_3)_{0.96}$
3. Coniferyl alcohol plus 0.47 H_2O	C_9	$H_{9.94}$	$O_{2.47}$	$(OCH_3)_{1.00}$
Difference (3. minus 2.)		$H_{1.57}$		$(OCH_3)_{0.04}$

CONFLICTING EVIDENCE

Pea roots, in the presence of hydrogen peroxide, converted eugenol into a ligninlike material (57-59). The experimental conditions were such as to favor the oxidation of eugenol to coniferaldehyde, but not the reduction of the aldehyde to the alcohol. In recent work with

hydrogen peroxide, a peroxidase, and filter paper as a growing site, eugenol was again converted into a ligninlike material (60,61). It was argued in this instance that the theory of coniferyl alcohol being responsible for lignin formation was untenable (5). Thus, it was suggested that, in addition to coniferyl alcohol, coniferaldehyde might also be responsible, to some extent, for the formation of lignin.

The important question as to where in the tree these lignin building stones are formed is still unanswered. It has been suggested that these units originate within the lignifying cells at a certain stage of their differentiation (62). However, lignifying tissues are not thought to have the capacity for such biosynthesis, and, therefore, these lignin building stones probably originate outside the zone of lignification (10).

It should also be noted that polymerization of the enzymatically synthesized dimers of coniferyl alcohol (cf. Figure 6), may occur non-enzymatically (47). This type of polymerization yields some alpha-beta coupling as shown in Figure 7 (5).

SUMMARY

The analytical and biosynthetic evidence suggests not only the general pathway shown in Figure 8 for the formation of the lignin building stones and their subsequent polymerization into lignin, but also the structural relationships for the gymnosperm lignin system shown in Figure 7 (5).

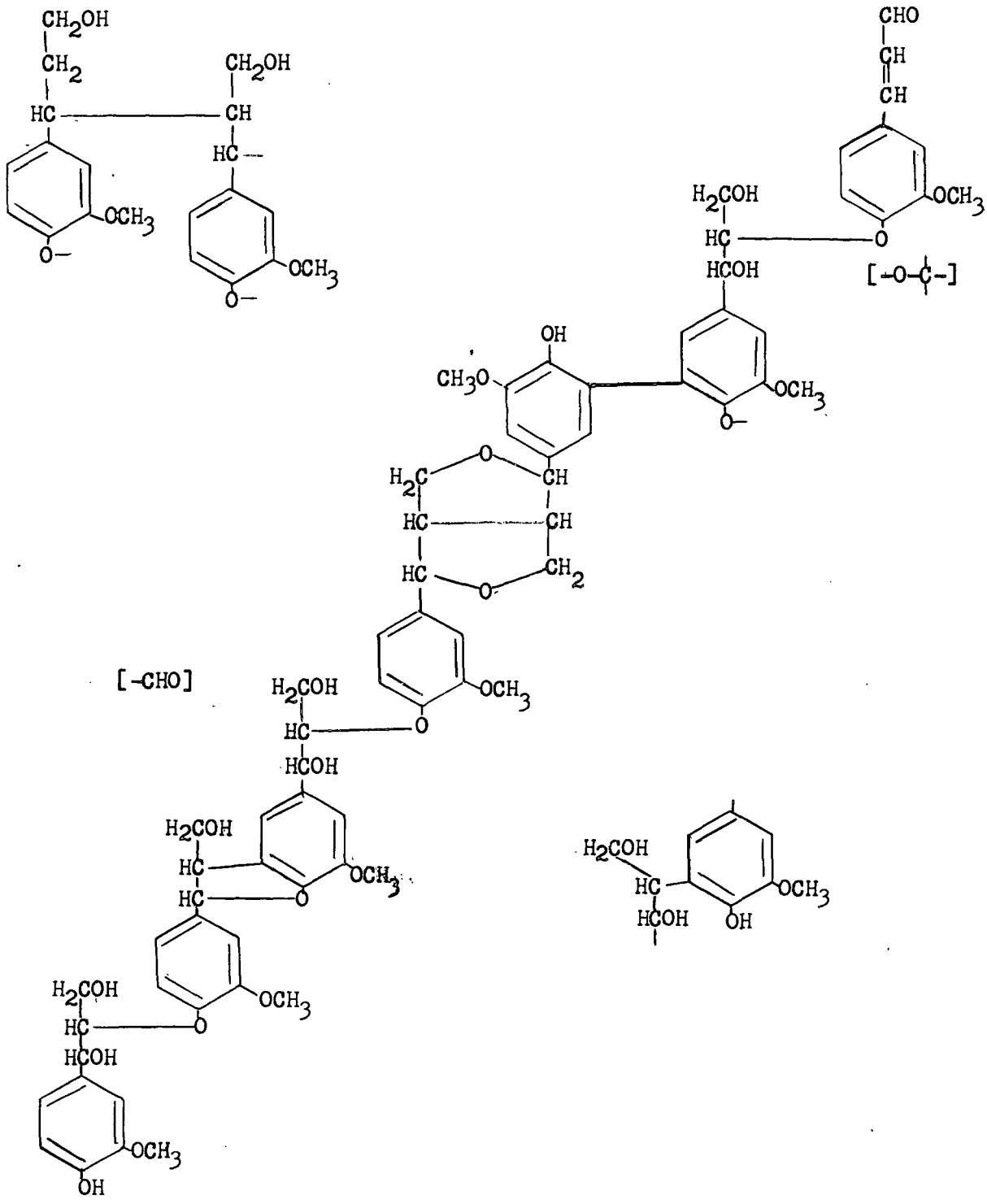


Figure 7. Structural Units Thought to be Present in Gymnosperm Lignin (5)

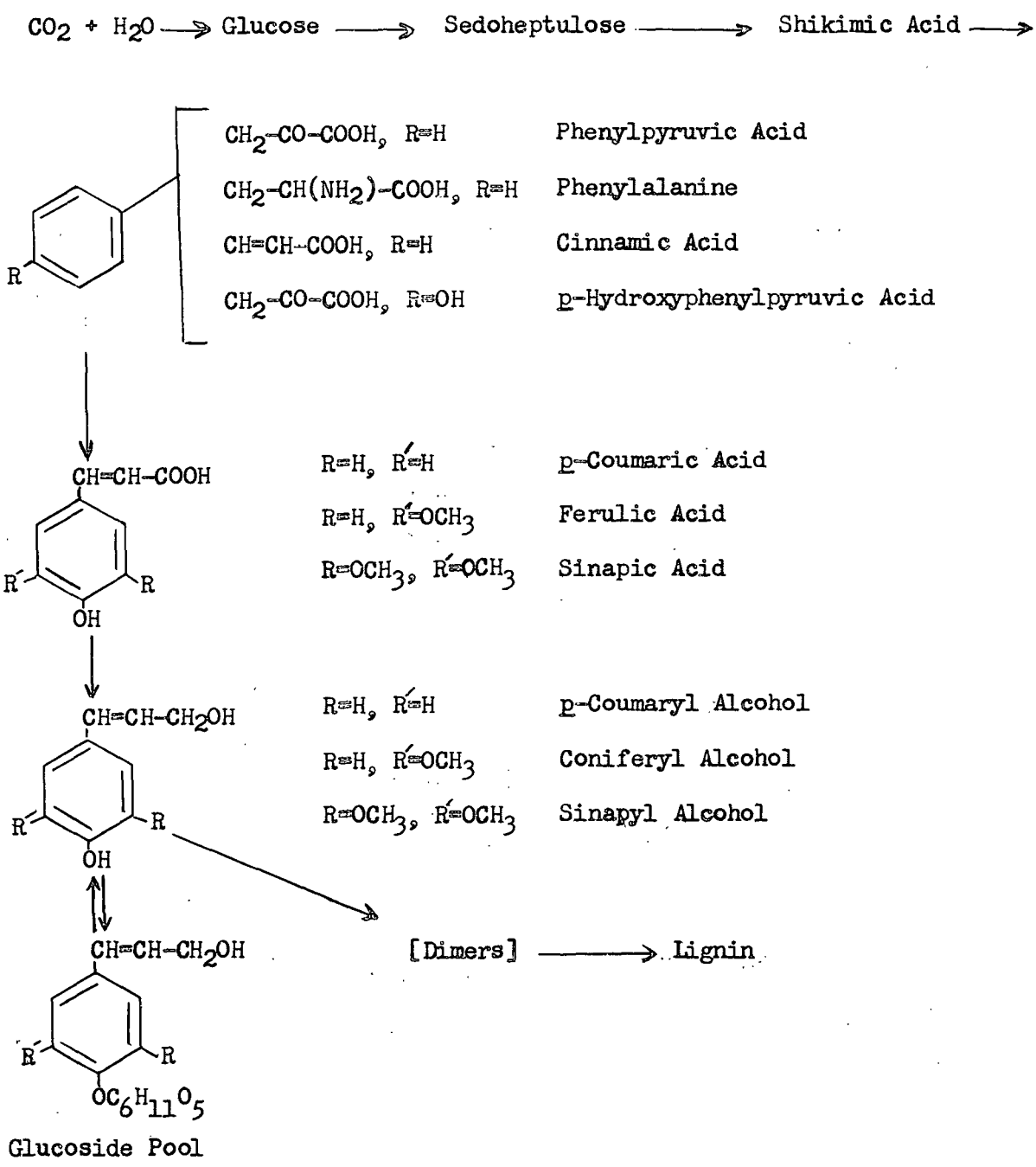


Figure 8. The General Biosynthetic Pathway to Lignification

The newer views of lignin formation are based almost entirely on work done with the relatively simple gymnosperm lignin system. Theories concerning the structure and biosynthesis of angiosperm mono- and dicotyledonous lignins have then been drawn largely by analogy from this gymnosperm lignin prototype.

PRESENTATION OF THE PROBLEM

The evidence presented previously resulted in a biochemical pathway leading to the synthesis of lignin whereby glucose is converted to the aromatic lignin building stones via the hydroaromatic acids. These lignin building stones are then protected by glucoside formation for transport from the sites of synthesis via the inner phloem, through the cambium and into the lignifying xylem, where they are freed and polymerized into lignin.

In a recent study of newly formed aspenwood[1] (63), the constituents extracted by methanol from several xylem tissues, collected at various stages of lignification, were considered for their relationships to the biosynthesis of lignin. Several low-molecular weight phenolic materials were found, and based on their chemical structures, they could be considered precursors to the lignin of aspenwood. However, no comparable investigation of aspen inner phloem has been made.

In xylem, the lignification of fibers is a prominent feature. The inner phloem has fewer lignified fibers, but is important in the transport and storage functions of the tree. It might be expected that certain of the lignin precursors would be more readily detectable if present as reserve matter in this tissue. Thus, this study was primarily concerned with the problems: Does the inner phloem of aspen contain low-molecular weight phenolic materials which support the theories concerning lignin formation?

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[1] Aspenwood is used here to mean Populus tremuloides only.

While designing the experimental program, it was realized that collecting the inner phloem would also provide material from the stone cell layer of aspen bark. In addition to answering questions concerning lignin biosynthesis, it was thought that collecting samples during the four seasons of the year might provide some information concerning any seasonal variation of materials present in these extracts[1]. Thus, two more objectives of this study arose: Are there any chemical differences between methanol extracts of the two phloem layers? Are there any qualitative differences between extracts collected during the four seasons of the year?

[1]It should be noted that tissue samples from enough trees were not obtained to draw any statistically valid conclusions regarding seasonal variation.

SUMMARY OF EXPERIMENTAL RESULTS

The inner phloem and stone cell layer of P. tremuloides trees, collected during the four seasons from fall, 1957 through summer, 1958, were extracted with absolute methanol at room temperature. The solids extracted were then fractionated according to standard procedures. Examination of these materials gave the following results:

1. The air-dried, methanol extracted inner phloem and stone cell layers for all seasons were Mäule-positive.

2. The crude extract from the stone cell layer for each season was chromatographically more complex than the corresponding extract from the inner phloem.

3. Paper chromatography revealed that all water-soluble fractions of inner phloem and stone cell layer contained Mäule-positive materials.

4. All ether-soluble fractions of inner phloem and stone cell layer contained no Mäule-positive materials by chromatographic analysis.

5. p-Hydroxybenzoic acid, p-coumaric acid, vanillic acid, pyrocatechol and benzoic acid have been isolated as crystalline compounds from the ether-soluble fractions of inner phloem. They were identified by their color reactions, R_f values, melting points and mixed melting points with authentic samples.

6. The ether-soluble fractions of inner phloem also yielded two unknown crystalline materials. These compounds have been partially characterized by their color reactions, R_f values, melting points, infrared and ultraviolet spectra.

7. Ferulic acid has been tentatively identified in various ether-soluble fractions of inner phloem by R_f values and color reactions in comparison with an authentic sample.

8. Paper chromatography of the ether-soluble fractions of inner phloem and stone cell layer gave no evidence for the presence of the following possible lignin precursors: sinapaldehyde, coniferaldehyde, p-coumaraldehyde and cinnamic acid.

9. Other compounds tested and not found were: syringaldehyde, vanillin, p-hydroxybenzaldehyde, syringic acid, protocatechuic acid, 2,5-dihydroxybenzoic acid, pyrogallol and resorcinol.

10. The water-soluble fraction of T-81,82 inner phloem (spring) spontaneously yielded a crystalline material which has been tentatively identified as salireposide by its color reactions, R_f values, melting point and infrared spectrum.

11. Paper chromatography revealed the presence of salireposide in all of the water-soluble fractions of inner phloem and stone cell layer.

12. The water-soluble fractions of inner phloem and stone cell layer gave no chromatographic evidence for syringin, coniferin, shikimic

acid or quinic acid.

13. Glucose, sucrose, fructose, salicin, populin and tremuloidin have been tentatively identified in all of the water-soluble fractions of inner phloem and stone cell layer by R_f values and color reactions in comparison with authentic samples.

14. Vanillic acid was found only in the ether-soluble fractions of T-74,75 inner phloem (fall) and T-81,82 stone cell layer (spring).

15. Ferulic acid was detected by paper chromatography in all ether-soluble fractions of inner phloem and stone cell layer except T-74,75 (fall).

16. Paper chromatography of the precipitates-from-water of inner phloem and stone cell layer revealed that Maüle-positive materials were present only in samples T-81,82 (spring) and T-88,89,90 (summer).

17. Wiesner-positive materials were detected in all of the water-soluble fractions of inner phloem and stone cell layer except T-88,89,90 inner phloem (summer) and T-74,75 inner phloem (fall).

18. Wiesner-positive materials were detected in all precipitates-from-water of inner phloem and stone cell layer except T-74,75 inner phloem (fall).

19. A new procedure of qualitative infrared spectral analysis of the various fractions, i.e., complex mixtures, was developed and found consistent with other qualitative analysis.

GENERAL METHODS

COLLECTION PROCEDURES

All experimental work was performed on two layers of bark from P. tremuloides trees cut from the Rhinelander Paper Company Industrial Forest, Eagle River, Wis. The tissue samples were collected at four intervals throughout a one-year growing cycle: mid-October, 1957, trees T-74 and 75; mid-February, 1958, trees T-76 and 77; mid-May, 1958, trees T-81 and 82; and mid-July, 1958, trees T-88,89 and 90. After the trees were felled and the branches removed, the stem was cut into short bolts for easy handling. A disk, cut from the base of each tree, was used for age and diameter measurements.

Tissue collections were made as soon after felling as possible. All tissues collected in the fall, spring and summer were obtained within three hours after the trees were felled. Due to the cold weather, the trees felled in winter were cut into bolts and sealed in polyethylene bags for transportation and storage out-of-doors. In this case, all tissue samples were collected within 4 days after felling. As soon as the tissues were removed from the bolt, they were immersed in absolute methanol to halt enzyme activity and to begin the extraction.

The techniques used to secure the two layers of bark tissues were as follows:

Stone Cell Layer. The outer bark was scraped from the bolts with a potato peeler and jackknife, making sure that all of the green-colored tissue was removed. The orange-brown tissue uncovered was the stone cell layer. This layer was removed with a spokeshave, cut into small pieces and placed in methanol. The entire stone cell layer of each bolt was not removed for experimental purposes. Since the inner phloem lay just inside this layer, care was taken to avoid inclusion of this tissue in the stone cell layer. Some carry-over occurred, however.

Inner Phloem. The bolt with part of the stone cell layer removed was then carefully cleaned of residual stone cell tissue with a jackknife. The tissue exposed was the cream-colored, highly moist, fibrous inner phloem. This tissue was very easily removed by two techniques depending on the season. In the fall and winter, the jackknife was placed nearly parallel to the surface of the bolt and drawn lengthwise along its surface. The inner phloem separated from the wood very easily and could always be differentiated from xylem in case of a deep cut by the differences in texture and color, the xylem being lighter in color and more dense than the inner phloem.

Since the yield of stone cell layer per bolt was always greater than inner phloem tissue, some bolts were used for inner phloem collection exclusively, and, in this respect, a slightly different procedure was employed in the spring and summer collection periods. After the tree was felled, the whole bark was stripped off and cut into long narrow strips. With the aid of a jackknife, the inner phloem was

peeled from these strips in long strands which were then cut into small pieces and immersed in methanol. Care was taken not to contaminate any of the inner phloem with stone cell tissue. A cross section of the bole of an aspen tree is shown in Figure 9.

EXTRACTION PROCEDURES

All extractions were carried out at room temperature. The methanol which was used at the time of collection was decanted after four days and fresh methanol added. Two additional solvent changes were made with the total extraction time being 14-16 days. The crude methanol extract was filtered through fluted paper to remove any fiber debris, and the extracted tissue was subsequently air dried for five days and weighed.

FRACTIONATION PROCEDURES

The methanol extracts were broken down into fractions according to the schemes of Mugg (63) and Sultze (65) with minor modifications. The methanol extracts were first concentrated to a water solution in a circulating steam evaporator. During this operation, a dark brown resinous material separated. The water was decanted and this precipitate washed and redissolved in methanol. The water solution was then exhaustively extracted with hexane followed by ether. In all cases, during the hexane extraction, extreme care was taken to avoid emulsion formation. The ether extraction was first attempted using a continuous liquid-liquid extractor, but resulted in emulsion formation and carry-over. However, the familiar separatory funnel procedure proved satisfactory. After

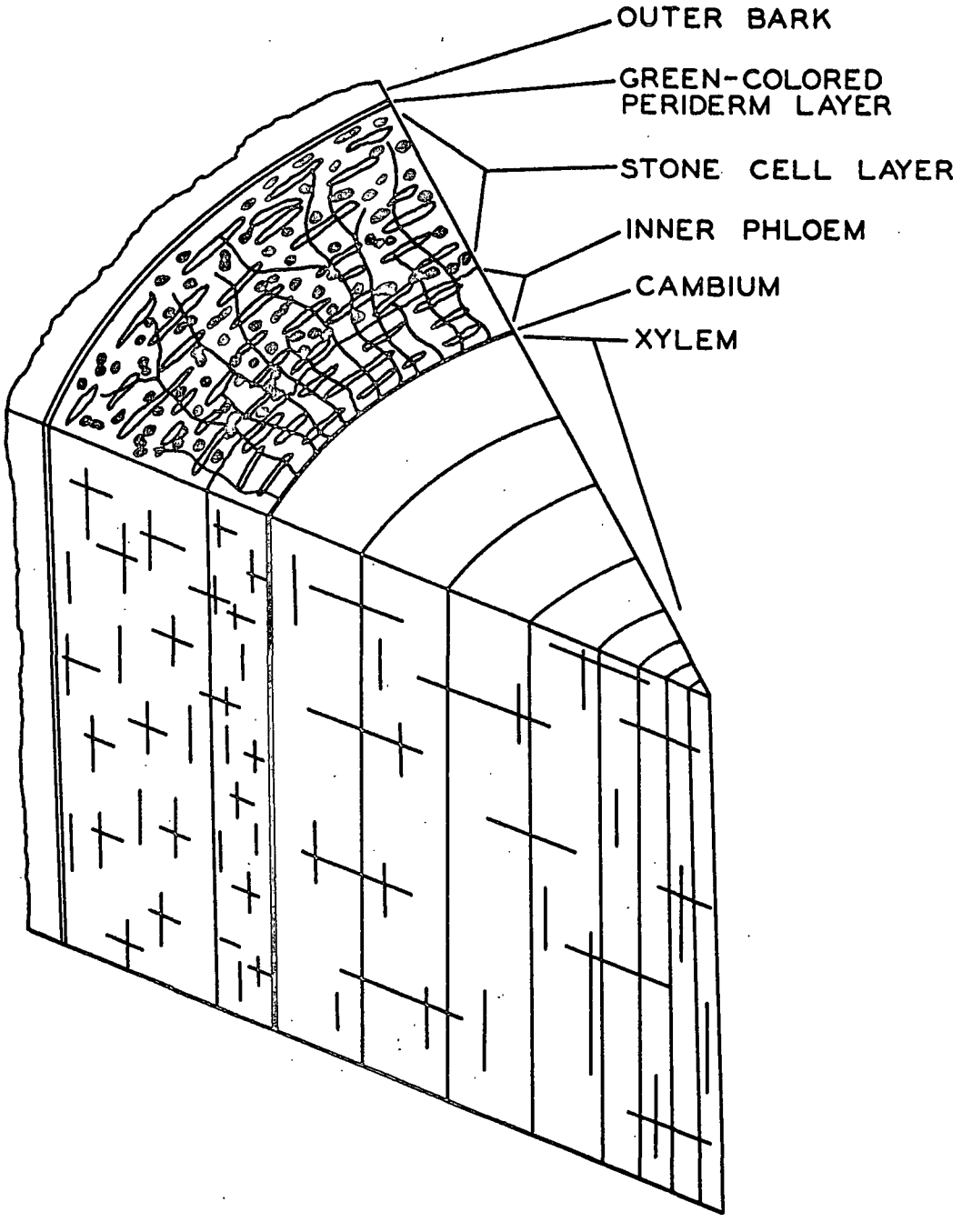


Figure 9. Cross Section of the Bole of an Aspen Tree, Adapted from (64)

concentration, the ether solutions also yielded small deposits of a gummy material similar in color to the precipitates-from-water. The ether was decanted and this precipitate redissolved in methanol. In all fractionations involving the use of separatory funnels, a minimum of eight extractions were performed.

Figure 10 represents the fractionation scheme employed in this work. Each fraction of T-81,82 inner phloem and stone cell layer was also subjected to an infrared spectral analysis. Figure 11 illustrates the infrared curves obtained from the first three major fractions of inner phloem, i.e., the precipitate-from-water, the hexane-soluble materials and the water-soluble materials. Details of the analytical treatment of these infrared data appear in the Appendix.

PAPER CHROMATOGRAPHY

Whatman No. 1 filter paper was employed for all paper chromatography unless mentioned otherwise. After their development, the papers were air dried and examined under ultraviolet light. When formic acid or acetic acid was used in the developing solvents, the air-dried chromatograms were also steamed and subsequently air dried prior to spraying. This mild steam treatment aided in removing the last traces of these acids which caused background difficulties with some of the qualitative spray reagents. For preparative purposes or ultraviolet spectral analysis, it is recommended that the papers be prepared in the following manner:

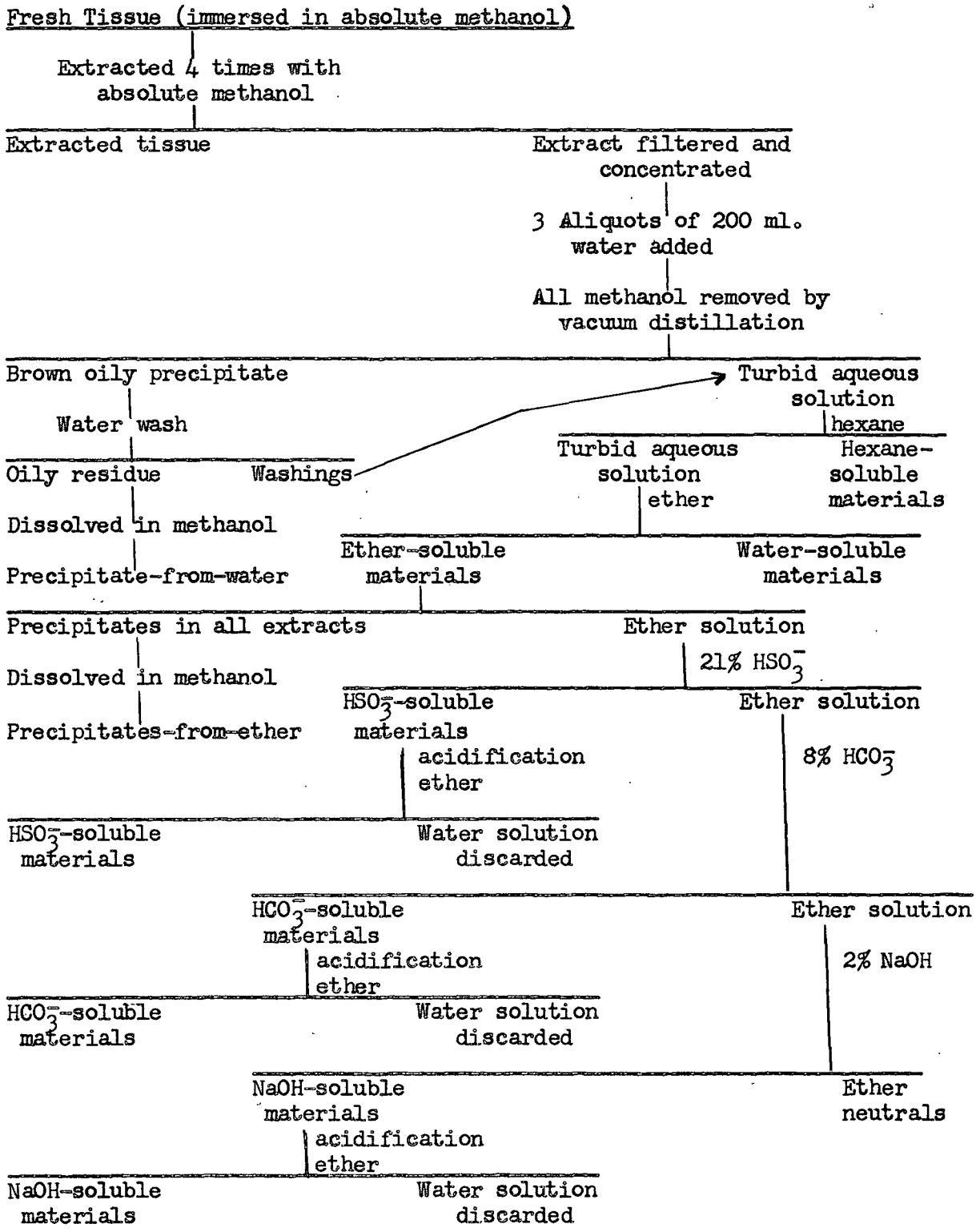


Figure 10. Flow Diagram Showing Preparation and Fractionation of Extracts

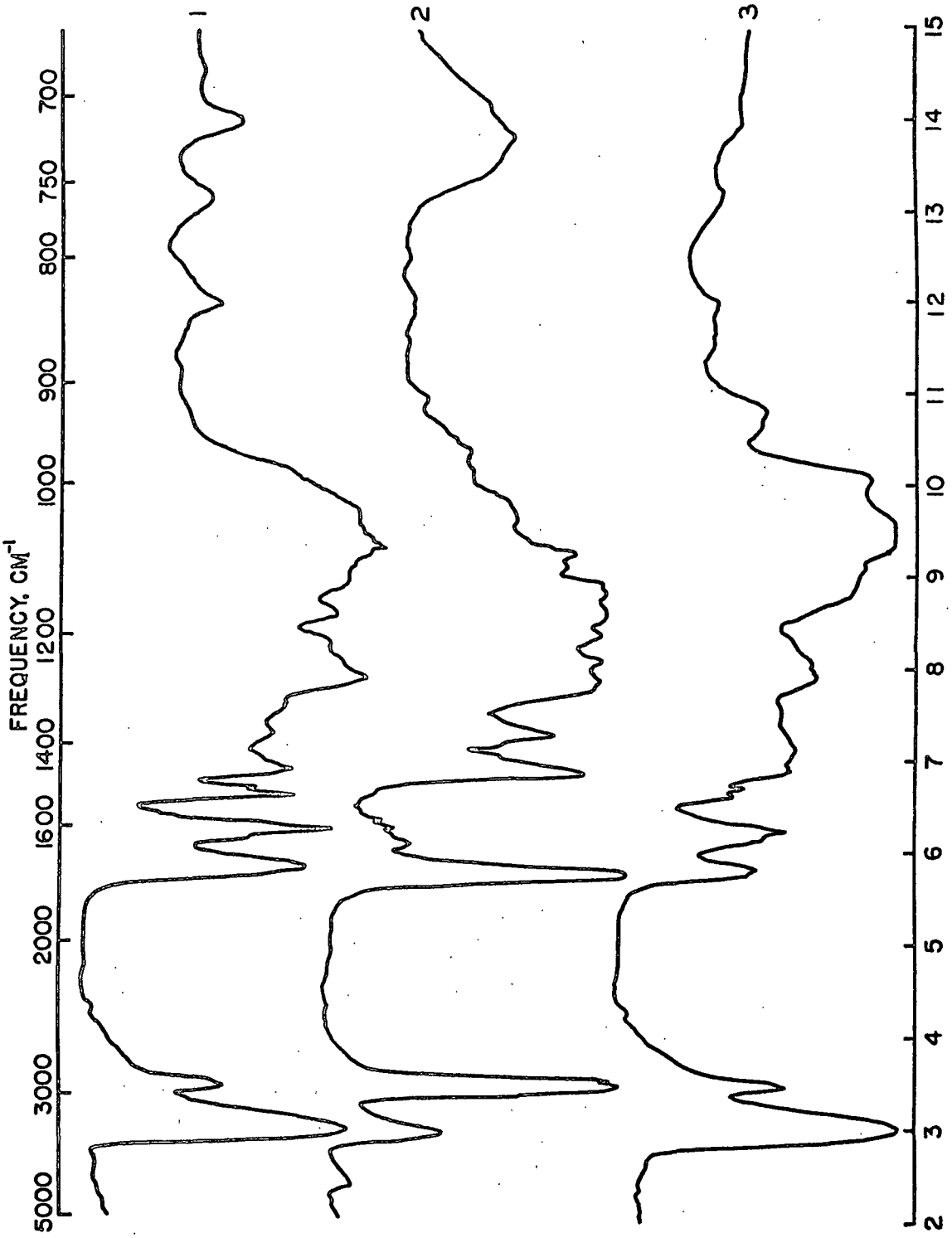


Figure 11. Infrared Spectra of the Precipitate-from-Water (1), the Hexane-Soluble Materials (2), and the Water-Soluble Materials (3). T-81,82 Inner Phloem

(1) Wash (descending chromatography) the paper with distilled water for 24 hrs.

(2) Air dry until cockling commences, approximately 3-4 hrs.

(3) Repeat the water wash with complete air drying, i.e., 6-8 hrs.

(4) Wash the paper with the eluting solvent until the solvent runs off the paper.

(5) Air dry.

(6) Repeat steps 4 and 5 with the developing solvent.

(7) Spot the papers and proceed as usual.

This procedure is a modification of that developed by Detrick (66).

The developers used for paper chromatography included:

(a) the upper phase of a toluene-acetic acid-water (4:1:5) mixture (TAW) (67);

(b) benzene saturated with 98% formic acid (BF) (68);

(c) n-butyl ether saturated with water (NBEW) (69);

(d) the upper phase of a n-butanol-acetic acid-water (4:1:5) mixture (BAW) (70);

(e) n-butanol saturated with 2% aqueous ammonia (BAm) (71);

(f) n-butanol saturated with an aqueous solution of 1.5N ammonia and 1.5N ammonium carbonate (BAmC) (72);

(g) the upper phase of a benzene-acetic acid-water (2:2:1) mixture (BzAW) (73);

(h) the upper phase of a n-butanol-ethanol-aqueous solution of 1.5N ammonia and 1.5N ammonium carbonate (40:11:19) mixture (BEAmC) (72);

(i) the upper phase of a n-butanol-pyridine-water (10:3:3) mixture (BPW) (74);

(j) a mixture of phenol-formic acid-water (3:0.01:1) (PFW) (75).

The qualitative spray reagents used to identify various materials on the paper chromatograms included:

(a) Wiesner reagent. Only the characteristic red-violet color was taken as positive, indicating a p-hydroxycinnamyl aldehyde structure (76,77). Other colors ranging from orange-red to yellow are produced by C_6-C_1 phenolic aldehydes such as syringaldehyde and p-hydroxybenzaldehyde. This reagent is a mixture of 1% phloroglucinol dissolved in 12% hydrochloric acid (78).

(b) Mäule test. The characteristic cerise color was shown to be rather specific for compounds containing the pyrogallol nucleus (79). After the chromatogram was developed and air dried, it was moistened with steam and placed in an atmosphere of chlorine for 5-10 minutes. The paper was then sprayed with a freshly prepared 10% solution of sodium sulfite. This test was actually developed by Cross and Bevan (80).

(c) p-Anisidine reagent (pA). This reagent gives characteristic green-brown colors with aldohexoses (81), and is a 0.5% solution of p-anisidine hydrochloride in n-butanol. After spraying the chromatogram with this reagent, the paper was placed in an oven at 105°C. for several minutes to develop the colors.

(d) 2,4-Dinitrophenylhydrazine reagent (DNPH). A saturated solution of 2,4-dinitrophenylhydrazine in 12% hydrochloric acid gives a yellow-orange

color with aldehydes and ketones (82). The 2,4-dinitrophenylhydrazones of aldehydes are usually deeper in color than those of ketones.

(e) Alcoholic ferric chloride (FC). A 2% solution of ferric chloride in absolute ethanol was used to detect phenols (83).

(f) Periodate-piperazine-nitroprusside reagent (PPN). This reagent was used to detect hydroaromatic acids. Shikimic acid and quinic acid appeared as yellow spots on a light purple background. The paper chromatogram is first sprayed with a saturated solution of sodium meta-periodate diluted with two volumes of water and allowed to air dry. An ethanolic solution of sodium nitroprusside and piperazine is then sprayed on the paper and heated at 105°C. for 5 minutes to develop the spots (84).

(g) Diazotized p-nitroaniline reagent (PNA). Most phenols will react to give bright characteristic colors ranging from brilliant red (p-hydroxy-benzoic acid) to Yale-blue (p-coumaric acid). Phenols having the syringyl nucleus with a para-substituted side chain, i.e., syringic acid, give very faint colors which are quite difficult to detect. The air-dried chromatogram is lightly sprayed with a solution of diazotized p-nitroaniline and allowed to air dry. The paper is then sprayed with a saturated solution of sodium carbonate (85).

(h) Silver nitrate reagent (SN). For the location of glycosides, the silver nitrate procedure (86) with a slight modification (87) was employed. The air-dried chromatogram is dipped in a solution of 3% silver nitrate prepared by dissolving 3 g. silver nitrate in 5 ml. water and adding 95 ml. acetone. A few drops of water may be added to re-dissolve the precipitated silver nitrate. The paper is then uniformly

sprayed with a 2% solution of sodium hydroxide in 95% ethanol. This solution is prepared by dissolving 2 g. sodium hydroxide in 5 ml. water and slowly adding 95 ml. absolute ethanol. The paper is allowed to air dry 5 to 10 minutes to develop the spots. The chromatogram is then dipped in a concentrated solution of sodium thiosulfate (350 g./l.), thoroughly washed with water and allowed to air dry. Sugars and glycosides appear as dense black spots on a nearly white background.

DETERMINATION OF INFRARED SPECTRA

All infrared spectral determinations were made by Mr. Lowell Sell of the Analytical Department of The Institute of Paper Chemistry with a Perkin-Elmer Model 21 Recording Infrared Spectrophotometer. When the material to be analyzed was in the solid phase, it was ground with potassium bromide and the mixture pressed into a transparent wafer for insertion into the spectrophotometer. If, on the other hand, the material were an oil, the potassium bromide wafer was first formed and the oil smeared over one of its plane surfaces prior to placing it in the instrument. The materials to be analyzed were dried in a vacuum desiccator containing calcium oxide and magnesium chlorate for at least 48 hrs.

DETERMINATION OF ULTRAVIOLET SPECTRA

All ultraviolet spectral determinations were made by Mr. Lowell Sell with a Beckman Model DK-2 ratio recording Spectrophotometer. The solvent was 95% ethanol.

SOLVENTS AND REAGENTS

All solvents and reagents used were reagent grade.

AUTHENTIC SAMPLES OF COMPOUNDS

Authentic samples of syringin and coniferin were supplied by Dr. R. E. Kremers, and a sample of shikimic acid was obtained from Dr. B. D. Davis. Dr. I. A. Pearl donated the following compounds: sinapaldehyde, p-coumaraldehyde, syringaldehyde, p-hydroxybenzaldehyde, ferulic acid, syringic acid, vanillic acid, p-hydroxybenzoic acid, populin and tremuloidin. Other compounds were obtained from commercial sources.

COLLECTION AND PREPARATION OF MATERIAL

COLLECTION OF TISSUE

Tissue samples representing the inner phloem and stone cell layer of P. tremuloides were collected at four intervals throughout a one-year growing cycle. The samples collected in the fall of 1957 represented the beginning of cambial dormancy; those collected in the winter of 1958 represented the dormant cambium, and the two samples collected in May and July, 1958, represented the early and significant cambial activity re-pectively.

Table II summarizes the biometric data.

TABLE II

BIOMETRIC DATA

Tree	Date Collected	Age, years	Butt Diameter, cm.	Height, feet
74	10-10-57	33	22.5	60
75	10-11-57	20	10.6	45
76	2-20-58	31	16.9	57
77	2-20-58	33	17.9	66
81	5-26-58	33	19.0	51
82	5-27-58	35	23.2	65
88	7-8-58	31	10.9	48
89	7-9-58	30	16.2	39
90	7-10-58	37	21.6	62

EXTRACTION OF TISSUE AND FRACTIONATION OF EXTRACTS

All tissue samples were extracted with methanol and fractionated according to the standard procedure. During the fractionation procedure the yields of the major fractions were determined. Tables III, IV and V summarize these data.

TABLE III

YIELDS OF MATERIALS EXTRACTED FROM INNER PHLOEM

Fraction	Season			
	Fall	Winter	Spring	Summer
Total methanol extractives, %[a]	10.4	22.8	20.7	32.0
Precipitate-from-water, %[a]	1.5	3.8	3.1	5.4
Hexane-soluble materials, %[a]	2.7	3.6	2.2	5.3
Ether-soluble materials, %[a]	0.4	0.8	1.8	1.5
Water-soluble materials, %[a]	5.8	14.6	14.2	19.9

[a]Calculated on basis of air-dry, extracted tissue

TABLE IV

YIELDS OF MATERIALS EXTRACTED
FROM THE STONE CELL LAYER

Fraction	Season			
	Fall	Winter	Spring	Summer
Total methanol extractives, %[a]	10.6	15.3	11.4	18.7
Precipitate-from-water, %[a]	2.6	4.5	4.3	8.1
Hexane-soluble materials, %[a]	1.4	1.5	1.2	2.1
Ether-soluble materials, %[a]	0.3	1.4	0.2	1.5
Water-soluble materials, %[a]	6.4	7.8	5.9	7.0

[a]Calculated on basis of air-dry, extracted tissue

TABLE V

WEIGHT OF AIR-DRY EXTRACTED TISSUE AND
CORRESPONDING YIELD OF
METHANOL EXTRACTIVES IN GRAMS

Sample	Season			
	Fall	Winter	Spring	Summer
Inner phloem:				
Tissue	831	394	875	701
Extractives	87	90	181	224
Stone cell layer:				
Tissue	359	895	1263	1021
Extractives	38	137	144	191

THE MÄULE TEST ON THE METHANOL-EXTRACTED TISSUES

After the inner phloem and stone cell layer tissues had been extracted with methanol, they were allowed to air dry and then weighed. A portion of each sample was subsequently soaked in water, chlorinated and finally treated with sodium sulfite. This Mäule test was positive for all of the samples collected and indicated the presence of the pyrogallol nucleus, especially the syringyl nucleus, in the methanol-insoluble lignin.

STUDY OF FRACTIONS

DIFFERENCES BETWEEN TREES

In order to determine whether there were any qualitative differences between the methanol-soluble materials collected from different trees, the various fractions of T-74 and T-75 inner phloem and stone cell layer were compared by paper chromatography using n-butanol-acetic acid-water (4:1:5) (BAW) (70) and toluene-acetic acid-water (4:1:5) (TAW) (67). A portion of each fraction was spotted on Whatman No. 1 paper and developed by the descending method. The locations of the spots were determined by visual and ultraviolet examination (UV) and by spraying with 2,4-dinitrophenylhydrazine (DNPH) (82), an alcoholic solution of ferric chloride (FC) (83), diazotized p-nitroaniline (PNA) (85), and by use of the Mäule (79) and Wiesner (78) test reagents.

On the basis of the developing solvents and qualitative tests described above, the two methanol extracts representing the inner phloem were quite similar. The methanol extracts representing the stone cell layers of the two trees were also quite similar though the water-soluble materials and the precipitates-from-water were more complex, i.e., exhibited more spots on paper chromatography, than the corresponding fractions of inner phloem. Therefore, it was decided that the slight differences between the methanol extracts from two trees did not warrant separate treatment, and in all ensuing sample collections, two extracts were obtained, one representing a combination of inner phloem tissue from the trees felled, the other representing a combination of the stone cell layers from the same trees.

THE WATER-SOLUBLE MATERIALS

PAPER CHROMATOGRAPHY

Orienting paper chromatography of these fractions indicated that BAW and n-butanol-pyridine-water (BPW) (74) were satisfactory developers. After developing, the chromatograms were sprayed with p-anisidine hydrochloride (pA) (81), silver nitrate (SN) (87), PNA and the Mäule and Wiesner test reagents. R_f values and color reactions of the materials present in these fractions appear in Tables VI and VII.

Table VIII summarizes paper chromatographic data of authentic compounds which were determined in conjunction with the data obtained in Tables VI and VII.

On the basis of this evidence, all of the water-soluble fractions of inner phloem and stone cell layer contain the following compounds: Compound A, sucrose; Compound B, glucose; Compound C, fructose; Compound D, salicin; Compound F, populin and Compound G, tremuloidin.

Since the Mäule-positive materials found in these fractions did not correspond to syringin, no further work was done to characterize the Mäule-positive materials found in the water-soluble fractions.

ISOLATION AND TENTATIVE IDENTIFICATION OF COMPOUND E

The water-soluble materials of T-81,82 inner phloem had been set aside under refrigeration for several months when it was noticed that small white burrs had begun to precipitate. After five months, these

TABLE VI

R_f VALUES AND COLOR REACTIONS OF COMPOUNDS FOUND IN ALL WATER-SOLUBLE FRACTIONS OF INNER PHLOEM AND STONE CELL LAYER

Developer: BAW

Spray R_f	0.08[A]	0.14[B]	0.19[C]	0.56[D]	0.67[E]	0.80[F]	0.84[G]
pA	+ (brown)	+ (brown)	+ (yellow)	-	-	-	-
PNA	-	-	-	-	+ (purple)	-	-
SN	+	+	+	+	+	+	+

Developer: BPW

Spray R_f	0.08[A]	0.13[B]	0.18[C]	0.55[D]	0.60[E]	0.74[F]	0.81[G]
pA	+ (brown)	+ (brown)	+ (yellow)	-	-	-	-
PNA	-	-	-	-	+ (purple)	-	-
SN	+	+	+	+	+	+	+

A, B, C etc. refer to the compounds present in these fractions.

crystals were collected by gravity filtration, washed with cold distilled water and recrystallized twice from hot water, yielding long colorless needles melting at 203-204°C. with an intermediate phase change occurring at 152-153°C.

A few crystals were dissolved in 95% ethanol, spotted on several sheets of paper and developed with BPW. The results of this chromatography appear in Table IX.

TABLE VII

R_f VALUES AND COLOR REACTIONS OF MATERIALS FOUND IN THE WATER-SOLUBLE FRACTIONS OF INNER PHLOEM AND STONE CELL LAYER

Developer: BAW

Season	R_f Values of Maüle-positive[a] Materials	
	Inner Phloem	Stone Cell Layer
Fall	0.55-0.75 (streak)	0.0; 0.55-0.75 (streak)
Winter	0.50-0.65 (streak)	0.50-0.65 (streak)
Spring	0.17; 0.50-0.65 (streak)	0.17; 0.50-0.65 (streak)
Summer	0.16; 0.50-0.75 (streak)	0.16; 0.50-0.75 (streak)

Season	R_f Values of Wiesner-positive[a] Materials	
	Inner Phloem	Stone Cell Layer
Fall	-	0.03
Winter	0.74-0.86 (streak)	0.74-0.86 (streak)
Spring	0.70-0.80 (streak)	0.70-0.80 (streak)
Summer	-	-

Developer: BPW

Season	R_f Values of Maüle-positive[a] Materials	
	Inner Phloem	Stone Cell Layer
Fall	0.12; 0.44-0.69 (streak)	0.10; 0.45-0.70 (streak)
Winter	0.12; 0.43; 0.49-0.71 (streak)	0.12; 0.39; 0.49-0.71 (streak)
Spring	0.12; 0.36; 0.46-0.80 (streak)	0.12; 0.36; 0.46-0.80 (streak)
Summer	0.11; 0.35-0.54 (streak)	0.11; 0.35-0.54 (streak)

TABLE VII (continued)

R_f VALUES AND COLOR REACTIONS OF MATERIALS FOUND IN THE WATER-SOLUBLE FRACTIONS OF INNER PHLOEM AND STONE CELL LAYER

Season	R_f Values of Wiesner-positive[a] Materials	
	Inner Phloem	Stone Cell Layer
Fall	-	0.02
Winter	0.66-0.77 (streak); 0.90	0.66-0.79 (streak)
Spring	0.0-0.70 (streak); 0.78	0.0-0.70 (streak); 0.89
Summer	-	-

[a] On the basis of the amount of material spotted on the chromatograms, the Mäule- and Wiesner-positive materials were quite dilute.

TABLE VIII

R_f VALUES AND COLOR REACTION OF AUTHENTIC COMPOUNDS

Compound	Spray pA	SN	PNA	R_f (BAW)	R_f (BPW)
Sucrose	+ (brown)	+	-	0.08	0.08
Glucose	+ (brown)	+	-	0.14	0.13
Fructose	+ (yellow)	+	-	0.19	0.18
Salicin	-	+	-	0.56	0.55
Populin	-	+	-	0.80	0.74
Tremuloidin	-	+	-	0.84	0.81

TABLE IX

PAPER CHROMATOGRAPHY OF
CRYSTALLINE COMPOUND E

Developer: BPW

R_f	0.70-0.72
Maule	-
Wiesner	-
FC	-
PNA	+ (purple)
SN	+
UV	-

Compound E was detected by chromatography in all of the water-soluble fractions of inner phloem and stone cell layer, though its R_f had been depressed to 0.60 using BPW and was 0.67 when developing with BAW (Table VI).

The relatively high melting point together with the chromatographic properties listed above suggested the possibility of a glycoside. Hydrolysis of a few crystals using 1N hydrochloric acid at 100°C. for 30 minutes yielded, on chromatography, glucose (pA,SN) and two other unidentified spots (PNA). Infrared spectral analysis of the crystals yielded a curve, shown in Figure 12, that was identical in all respects with an infrared spectrum of a crystalline material recently isolated from the whole bark of aspen by Pearl, who has tentatively identified this compound as salireposide (88,89).

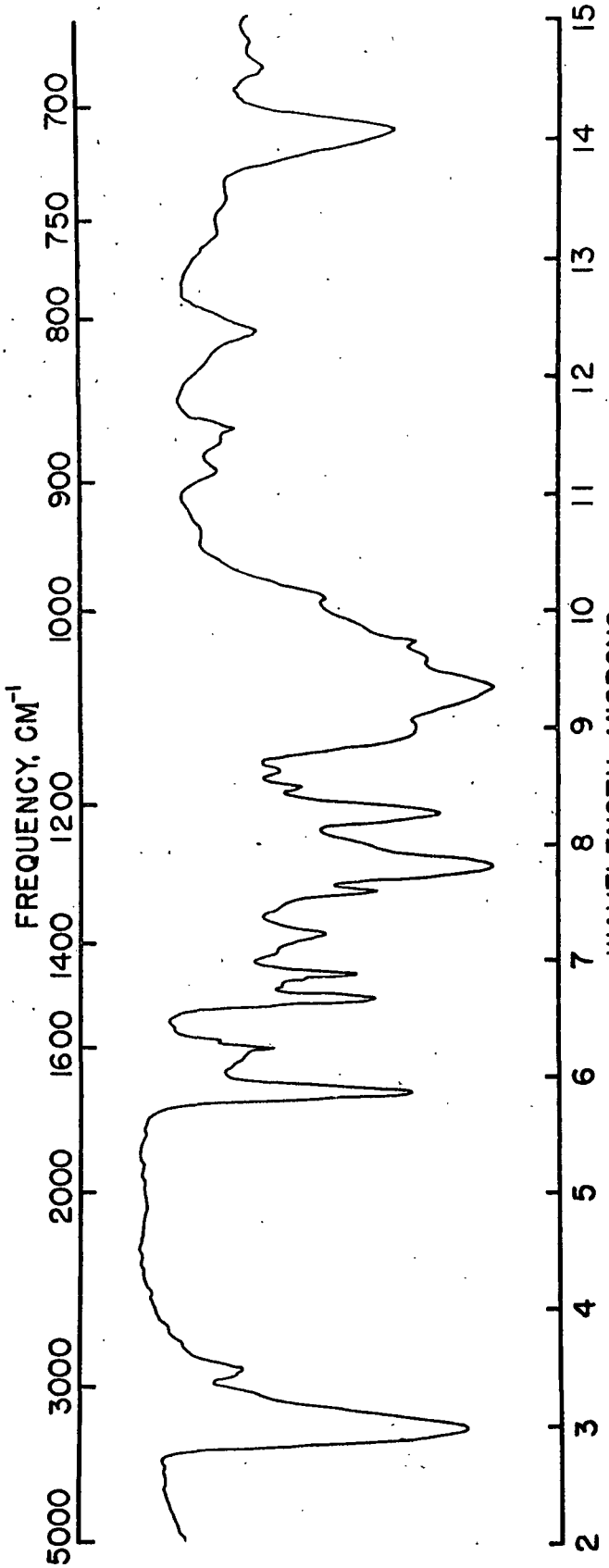
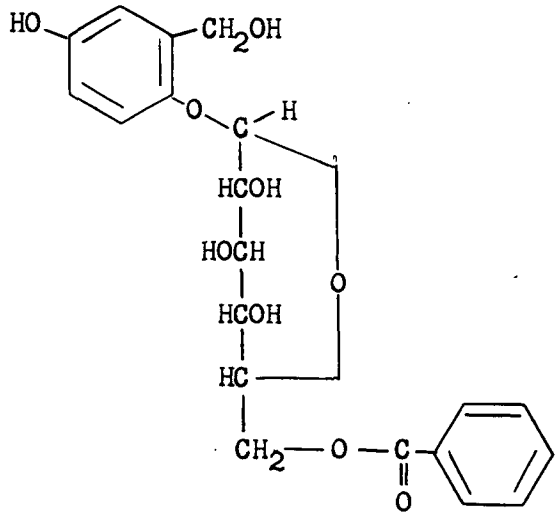


Figure 12. Infrared Spectrum of Suspected Salireposide



Salireposide

THE HYDROAROMATIC ACIDS

The water-soluble fractions of inner phloem and stone cell layer were also examined for hydroaromatic acids using paper chromatography. The developing solvent was a mixture of phenol-formic acid-water (3:0.01:1) (PFW) (75), and the spray reagents used to detect these materials were periodate-piperazine-nitroprusside (PPN) (84) and SN. After developing, the chromatograms were allowed to air dry until the smell of phenol had disappeared, usually six days. The papers were then sprayed with PPN and heated at 105°C. for 5 minutes to develop the colors. All of the water-soluble fractions of inner phloem and stone cell layer gave negative results. A sample of authentic shikimic acid was found at R_f = 0.60 and a sample of authentic quinic acid was detected at R_f = 0.50, both compounds giving the characteristic yellow-green color with PPN.

THE ETHER-SOLUBLE MATERIALS

The ether-soluble materials were fractionated by standard methods yielding the following fractions: bisulfite-soluble materials, bicarbonate-soluble materials, sodium hydroxide-soluble materials and ether neutrals.

PAPER CHROMATOGRAPHY OF THE BISULFITE-SOLUBLE MATERIALS

The bisulfite-soluble materials were analyzed by paper chromatography using n-butanol - 2% aqueous ammonia (BAm) (71), n-butyl ether saturated with water (NBEW) (69), and benzene-acetic acid-water (2:2:1) (BzAW) (73). The chromatograms were sprayed with DNPH, FC, PNA, the Mäule and Wiesner test reagents and examined under ultraviolet light.

The NBEW developer was discarded because the other two developers exhibited better resolution. Table X summarizes the chromatographic date obtained for all of the bisulfite-soluble materials from inner phloem and stone cell layer. Since these data were taken at various times throughout the year, it was found that the R_f values of these materials varied considerably, probably due to temperature fluctuations in the laboratory. Therefore, pyrocatechol, R_{py}, was used as a reference compound during the chromatography of the bisulfite-soluble materials.

ISOLATION OF CRYSTALLINE COMPOUND H

During the standard fractionation procedure involving bicarbonate, all of the bicarbonate-soluble materials of T-81,82 inner phloem were

TABLE X

R_{py}[a] VALUES AND COLOR REACTIONS OF THE MATERIALS PRESENT IN THE BISULFITE-SOLUBLE FRACTIONS OF INNER PHLOEM AND STONE CELL LAYER

Developer: BAm

Spray	R_{py}	0.10[H]	0.78[I]	1.00[J]	1.05[K]	1.10[L]
FC		-	-	+ (blue)	+ (blue)	-
Maüle		-	-	-	-	-
Wiesner		-	-	-	-	-
PNA		+ (blue)	+ (yellow-green)	+ (purple)	+ (green)	-
DNPH		-	+ (yellow)	-	+ (yellow)	-
UV		-	-	d	-	l

Developer: BzAW

Spray	R_{py}	0.07[H]	0.49-0.77[I]	1.00[J]	1.84[K]	2.21[L]
FC		-	-	+ (blue)	+ (blue)	-
Maüle		-	-	-	-	-
Wiesner		-	-	-	-	-
PNA		+ (blue)	+ (yellow-green)	+ (purple)	+ (green)	-
DNPH		-	+ (yellow)	-	+ (yellow)	-
UV		-	-	d	-	l

[a] R_{py}---reference pyrocatechol
H, I, J, etc. refer to compounds present
d---dark fluorescence
l---light fluorescence

contained in approximately 1 l. ether. This ether solution was dried over sodium sulfate and concentrated for chromatographic analysis. However, soon after the total volume reached approximately 400 ml., a white granular precipitate began separating. The volume was reduced to 200 ml. and the solids allowed to settle. The clear supernatant solution was decanted, and the solids were redissolved, with difficulty, in 600 ml. ether and again dried over sodium sulfate.

The resulting ether solution was concentrated under vacuum to approximately 25 ml. and allowed to stand several days under refrigeration. The clear solution yielded not more than 5 mg. (estimated) of small white burrs melting at 176-178°C. Cooling and remelting gave the same melting point. Despite this relatively sharp melting point, there was some apparent contamination because the walls of the melting point tube were lightly coated with a light brown substance after the bulk of material had melted into a clear, colorless liquid.

A few crystals were dissolved in 95% ethanol and chromatographed using BzAW. The R_f and R_{py} values and color reactions were identical to Compound H which was found in small amounts in the bisulfite-soluble fractions, (Table X). Because of the very small quantity of crystalline material isolated, no further work was done to characterize it more fully.

SEPARATION OF COMPOUNDS I, J, K AND L

Paper chromatography of the bisulfite-soluble fractions revealed that it was possible to separate Compounds I, J, K and L (Table X).

Compound J gave a blue color with FC and a purple color with PNA and had identical color reactions and the same movement characteristics in BzAW and BAm as authentic pyrocatechol.

In order to isolate enough of each material for possible positive identification, Whatman 3MM paper was employed to separate these materials. The paper was carefully prepared by washing (descending chromatography) with distilled water for 24 hrs., air drying, washing again with distilled water for 24 hrs. and air drying, and finally washing with 95% ethanol for 24 hrs. After the paper had air dried, it was stored in a sealed polyethylene bag until needed. Twenty papers were streaked with the ether solution of the bisulfite-soluble fractions of T-81,82 and T-88,89, 90 inner phloem and developed for 5 hrs. using BzAW. The materials were located on the chromatogram both by ultraviolet examination and by cutting guide strips and spraying with PNA. It was found that, using 3MM paper, Compounds I and J had overlapped. Therefore, the areas containing the mixture of Compounds I and J, Compound K and Compound L were separately extracted with 95% ethanol using a Soxhlet extractor. In order to prevent any decomposition of the pyrocatechol due to oxidation, the chromatograms were not allowed to air dry completely, the residual acetic acid being sufficient to keep the resulting ethanolic solution slightly acidic.

The alcoholic solution containing Compounds I and J was first filtered through Whatman No. 50 paper to remove any debris and then concentrated at 20°C. under vacuum to dryness. Since Compound I was probably water insoluble, i.e., a ketone or aldehyde, water-soluble

pyrocatechol was separated from Compound I by adding 30 ml. water to the dry solids. The solids were scraped from the walls of the flask with a spatula and the mixture was allowed to stand for several hours with intermittent stirring. The undissolved solids were filtered on paper and redissolved in 95% ethanol. This solution contained Compound I and was FC-negative. The filtrate from this manipulation contained pyrocatechol and was FC-positive.

ISOLATION AND CHARACTERIZATION OF COMPOUND I

The alcoholic solution containing Compound I was carefully concentrated at 20°C. under vacuum to dryness and the residue dissolved in 100 ml. ether. This ether solution was concentrated under vacuum until cloudy (40 ml.), and allowed to stand under refrigeration for several days. The solution yielded not more than 20 mg. (estimated) of colorless spheres melting sharply at 192-193°C.

A few crystals were redissolved in ether, chromatographed using BzAW, and sprayed with PNA. This test revealed that Compound I was chromatographically pure. Ultraviolet and infrared spectral analyses were then performed, and these curves are shown in Figures 13 and 14. The ultraviolet spectrum revealed a minimum at 256 mmu and a maximum at 273 mmu with no alkaline shift. The infrared spectrum suggested the possibility of two carbonyl groups.

A few milligrams were then hydrolyzed with 1N hydrochloric acid at 100°C. for 30 minutes. Absolute alcohol was then added to this

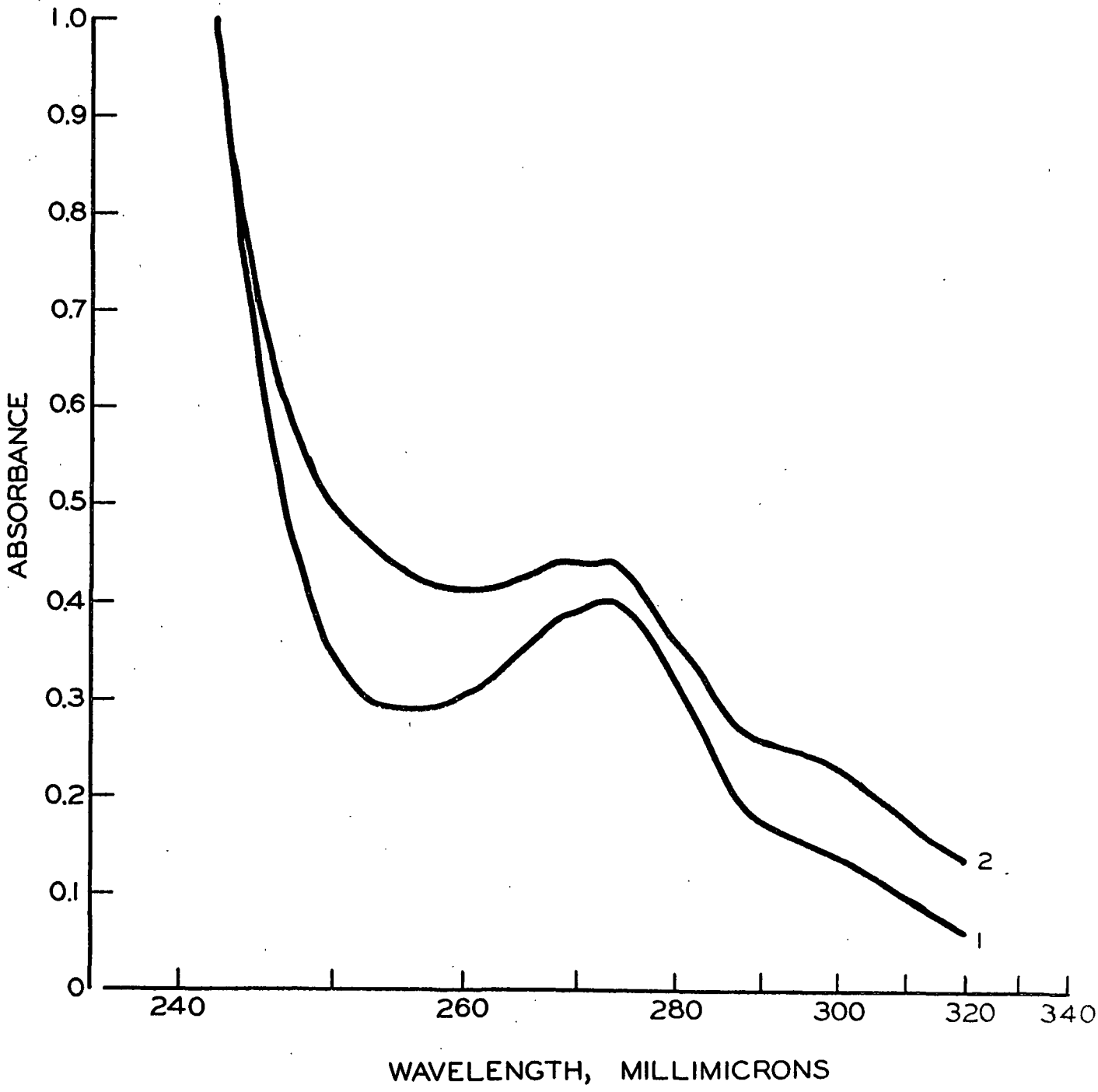


Figure 13. Ultraviolet Spectra of Compound I, Neutral Solution (1), Alkaline Solution (2)

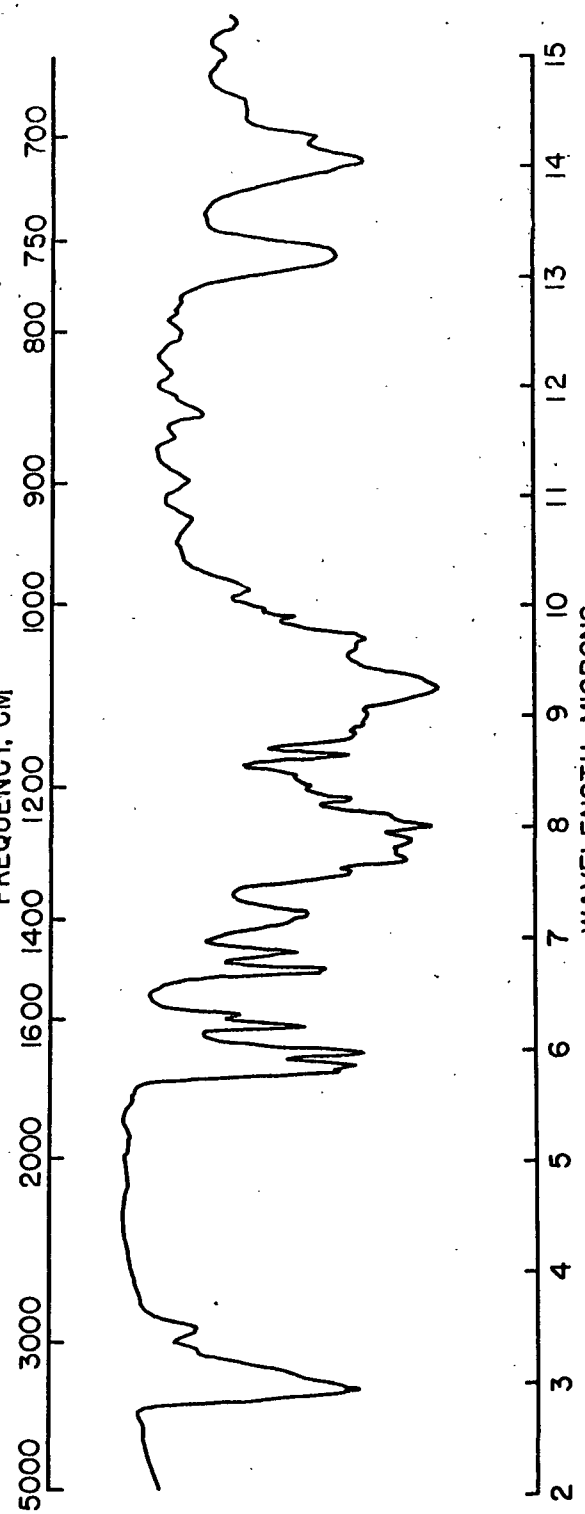


Figure 14. Infrared Spectrum of Compound I

aqueous solution to dissolve the colorless precipitate which had formed. Chromatography of this hydrolyzate gave the results appearing in Table XI.

TABLE XI

CHROMATOGRAPHY OF THE HYDROLYZATE OF COMPOUND I

Developer: BPW

Spray	R_f	0.05	0.15	0.22	0.87
PNA		-	-	+ (red-purple)	+ (orange)
SN		+	+	+	-
pA		+	+	-	-
FC		-	-	-	-
DNPH		-	-	-	-
UV (short)		-	-	-	l

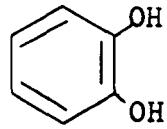
l --- light fluorescence

Authentic glucose, when chromatographed in BPW, has an R_f= 0.15 and reacts with SN and pA. Therefore, Compound I appears to be a glucoside. The two phenolic materials reacting with PNA are still unidentified. No further work was done with this material.

ISOLATION AND IDENTIFICATION OF PYROCATECHOL

The water solution containing Compound J, pyrocatechol, was carefully concentrated at 20°C. under vacuum to dryness. The dry solids were then dissolved in 2 ml. warm benzene. The benzene solution was

filtered through cotton and the filtrate concentrated to 1 ml. by heating. On slow cooling, the solution yielded light yellow needles which melted at 102-104°C. and did not depress a mixed melting point with authentic pyrocatechol.



Pyrocatechol

COMPOUND K

The alcoholic solution containing Compound K was carefully concentrated at 20°C. under vacuum to dryness. A preliminary test revealed that this material was recrystallizable from water. Therefore, 15 ml. water were added and the solution heated on a steam bath until all of the solids had dissolved. On slow cooling, however, the solution yielded a brown amorphous floc. This precipitate was concentrated by centrifugation, and washed twice with water. Because of the brown color and the fact that it tested positive to FC, decomposition was suspected. Actually, the major portion remained as a resinous mass even after warming with 95% ethanol. No further experimentation was done with Compound K.

COMPOUND L

During the standard fractionation procedures, the odor of oil of wintergreen became quite predominant in the various bisulfite-soluble fractions. While separating Compounds I, J and K by paper chromato-

graphy, it was noticed that a very strong fluorescent band at R_{py} = 1.10 and R_{py} = 2.21, using BAm and BzAW, respectively, gave the same odor (Table X). Therefore, this area was also extracted with 95% ethanol, filtered, and a few pellets of sodium hydroxide added. The solution was allowed to stand at room temperature for nine days with intermittent stirring. The solution was then acidified and carefully concentrated to a water solution. Trace amounts of a colorless precipitate formed after the ethanol was removed. The precipitate was separated from the water by an ether extraction. The ether solution was concentrated by heating and used for chromatography. Developing in BzAW, the material had an R_f = 0.41, gave a red color with FC and a reaction with PNA. Salicylic acid was suspected but not found, i.e., salicylic acid has an R_f = 0.87 in BzAW. No further work was done with Compound L.

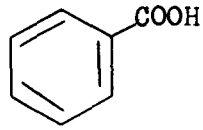
ISOLATION AND IDENTIFICATION OF BENZOIC ACID

During the standard fractionation procedures involving bisulfite, the step requiring acidification of the water solution of bisulfite always resulted in a strong odor of oil of wintergreen plus a precipitate. This odor and precipitate were subsequently extracted with ether and carried along with the general chromatographic analyses which followed. However, in the case of T-76,77 inner phloem, the precipitate was allowed to settle, the acidified water solution decanted and 95% ethanol added to dissolve the residue.

The ethanolic solution was concentrated to dryness, resulting in a viscous oil which still had a very strong odor of oil of wintergreen.

The flask containing this material was set aside for several months. On reexamining the flask, it was discovered that extremely long, colorless needles were growing from the walls of the flask by sublimation.

The infrared spectrum of these crystals was identical with a spectrum of benzoic acid (Figure 15), except for bands at 1249 $cm.^{-1}$ and 767 $cm.^{-1}$ A few crystals were placed in a capillary tube and resublimed. The melting point of the resublimed crystals was 119-120°C. When mixed with an authentic sample of benzoic acid, the melting point was 120-121°C. Authentic benzoic acid melts at 122°C.



Benzoic Acid

PAPER CHROMATOGRAPHY OF THE BICARBONATE-SOLUBLE MATERIALS

The bicarbonate-soluble materials were analyzed by paper chromatography with BzAW, n-butanol saturated with a buffer solution of 1.5N ammonia and ammonium carbonate (BAmC) (72), and the upper phase of a mixture of n-butanol-ethanol-1.5N ammonia and ammonium carbonate (40:11:19) (BEAmC) (72). The chromatograms were sprayed with PNA, FC, the Maüle test reagent and examined under ultraviolet light. Table XII summarizes the chromatographic data obtained for all of the bicarbonate-soluble materials from inner phloem and stone cell layer.

Paper chromatography of authentic samples with the above developers showed that the bicarbonate-soluble fractions contained: p-hydroxybenzoic

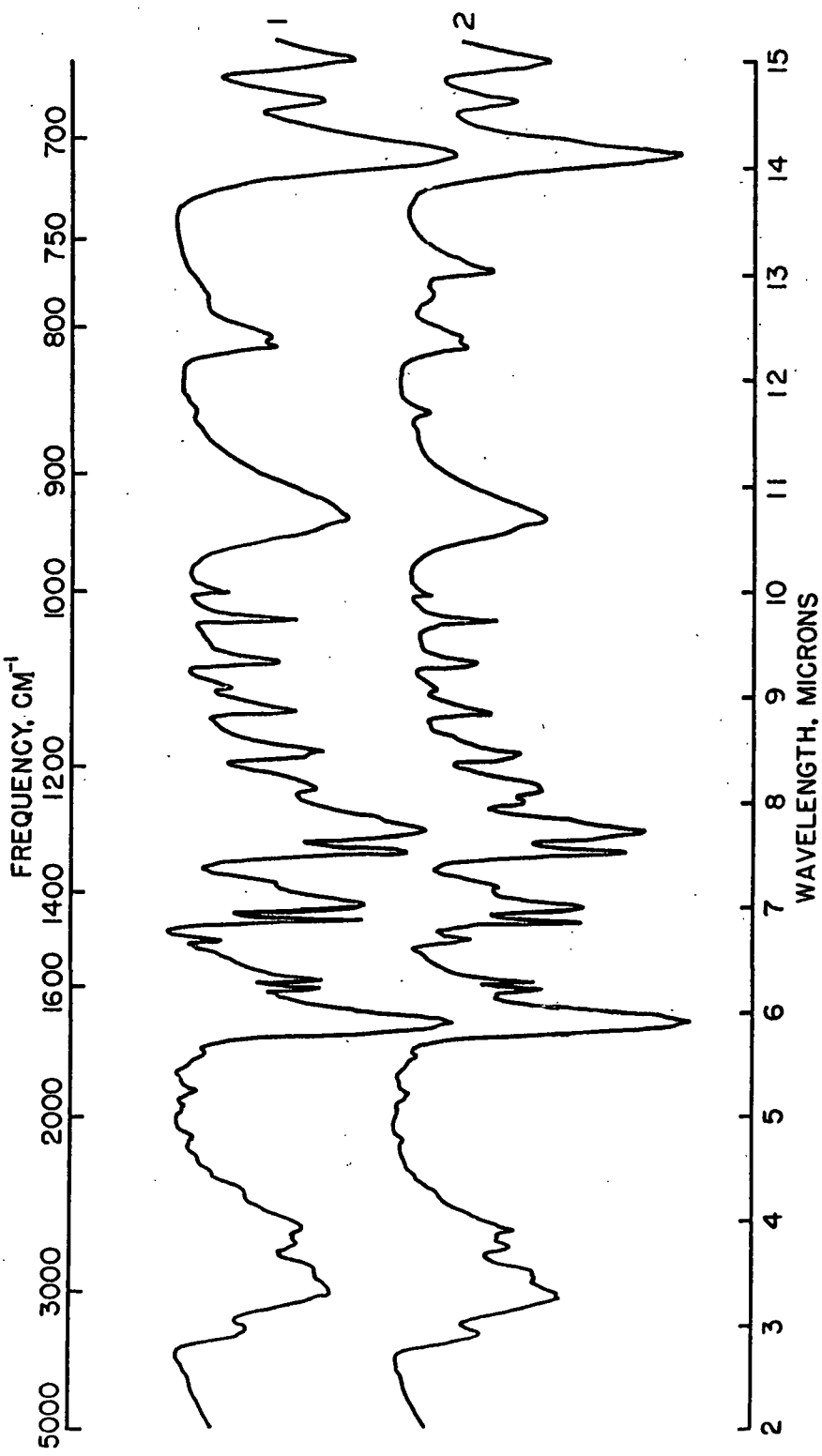


Figure 15. Infrared Spectra of Authentic Benzoic Acid (1) and Isolated Benzoic Acid (2)

TABLE XII

R_{va} VALUES AND COLOR REACTIONS OF THE MATERIALS PRESENT IN THE BICARBONATE-SOLUBLE FRACTIONS OF INNER PHLOEM AND STONE CELL LAYER

Developer: BzAW

Spray	R_{va}[a]	0.62[M]	0.65[N]	1.00[O]	1.03[P]
FC		-	+ (orange)	-	-
Mäule		-	-	-	-
PNA		+ (red)	+ (blue)	+ (purple)	+ (gray)
UV		d	d	-	l

Developer: BAmC[b]

Spray	R_{va}	1.00[O]	1.23[M]	1.63[N]
FC		-	-	+ (orange)
Mäule		-	-	-
PNA		+ (purple)	+ (red)	+ (blue)
UV		-	d	d

Developer: BEAmC

Spray	R_{va}	1.00[O]	1.25[M]	1.32[P]	1.67[N]
FC		-	-	-	+ (orange)
Mäule		-	-	-	-
PNA		+ (purple)	+ (red)	+ (gray)	+ (blue)
UV		-	d	l	d

[a]Reference vanillic acid

[b]Compound P not determined using this developer

M, N, O and P refer to materials present

d---dark fluorescence

l--light fluorescence

acid, Compound M; p-coumaric acid, Compound N; vanillic acid, Compound O; and ferulic acid, Compound P. These compounds were present in all of the bicarbonate-soluble fractions of inner phloem and stone cell layer with the following exceptions: ferulic acid was absent in T-74, 75 inner phloem and stone cell layer; vanillic acid was present only in T-74,75 inner phloem and T-81,82 stone cell layer.

SEPARATION OF COMPOUNDS M, N, AND O

Since the bicarbonate-soluble fraction of T-74,75 inner phloem contained only three compounds previously identified by paper chromatography as vanillic, p-coumaric and p-hydroxybenzoic acids, positive identification of these compounds was attempted.

Four sheets of Whatman 3MM paper were thoroughly washed with BzAW. The papers were then allowed to air dry and then streaked with the bicarbonate-soluble fraction of T-74,75 inner phloem. The chromatograms were developed in BzAW for seven hours. Guide strips were cut and sprayed with PNA. Vanillic acid had been completely separated at R_f= 0.70, while p-hydroxybenzoic acid and p-coumaric acid overlapped at R_f= 0.43 and R_f= 0.45, respectively. The sections of the chromatograms containing vanillic acid and the mixture of p-hydroxybenzoic acid and p-coumaric acid were cut out and eluted with 95% ethanol using a Soxhlet extractor.

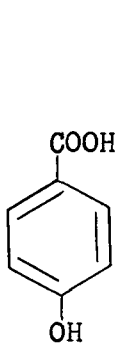
The alcoholic solution containing the mixture of p-hydroxybenzoic acid and p-coumaric acid was concentrated to a few milliliters and

spotted on four sheets of Whatman 3MM paper which had been previously washed with TAW. These chromatograms were developed with TAW for 20 hrs. Guide strips were cut and sprayed as before. Complete separation of the two acids was accomplished, i.e., p-coumaric acid -$R_{\text{coumaric acid}}$= 1.00; p-hydroxybenzoic acid -$R_{\text{coumaric acid}}$= 0.64. The sections of the chromatograms containing the two acids were cut out and eluted with 95% ethanol using a Soxhlet extractor. The three alcoholic solutions were subsequently treated in the same manner.

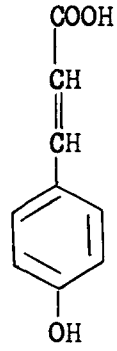
ISOLATION AND IDENTIFICATION OF VANILLIC ACID, p-HYDROXYBENZOIC ACID AND p-COUMARIC ACID

The alcoholic solution of each acid was concentrated to dryness and the residue extracted with ether. The ether solution was decanted and evaporated to dryness. The residue was again extracted with ether, decanted and evaporated to dryness. This procedure was repeated two times. The ether-soluble solids were then dissolved in hot water, filtered through cotton and concentrated to dryness. This step was repeated twice. After the final filtration, the hot water solution was placed on a clean watch glass. As the water cooled, crystallization began. The crystals were gently brushed to one side of the watch glass as the water solution evaporated. The solution containing Compound M yielded flat colorless platelets which melted at 212-214°C. and did not depress a mixed melting point with authentic p-hydroxybenzoic acid. The solution containing Compound N yielded long colorless needles which melted at 209-213°C. and did not depress a mixed melting point with authentic p-coumaric acid. The solution containing Compound O yielded

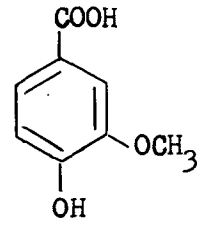
slightly yellow spheres which melted at 205-207°C. and did not depress a mixed melting point with authentic vanillic acid.



p-Hydroxybenzoic Acid



p-Coumaric Acid



Vanillic Acid

PAPER CHROMATOGRAPHY OF THE SODIUM HYDROXIDE-SOLUBLE MATERIALS

The sodium hydroxide-soluble materials were analyzed with benzene saturated with 98% formic acid (BzF) (63) and BEAmC. The chromatograms were sprayed with FC, PNA, the Mäule test reagent and examined under ultraviolet light. The results of these analyses appear in Table XIII. In addition to pyrocatechol, p-coumaric acid and ferulic acid reported in Table XIII, there were at least three additional unidentified materials which reacted with PNA and had very slow movement properties in the developing solvents used.

PAPER CHROMATOGRAPHY OF THE ETHER NEUTRALS

When these materials were chromatographed with TAW and BAW, no materials could be detected with FC, PNA, or the Mäule test reagent. Materials reacting with DNPH and the Wiesner test reagent were detected

TABLE XIII

R_{py} VALUES AND COLOR REACTIONS OF THE MATERIALS PRESENT IN THE SODIUM HYDROXIDE-SOLUBLE FRACTIONS OF INNER PHLOEM AND STONE CELL LAYER

		Developer: BzF		
Spray	R_{py}	1.00[J]	1.27[N]	3.50[P]
FC		+ (blue)	+ (orange)	-
Maule		-	-	-
PNA		+ (purple)	+ (blue)	+ (gray)
UV		d	d	l
		Developer: BEAmC		
Spray	R_{py}	0.26[P]	0.34[N]	1.00[J]
FC		-	+ (orange)	+ (blue)
Maule		-	-	-
PNA		+ (gray)	+ (blue)	+ (purple)
UV		l	d	d

J, N and P refer to compounds present, i.e., pyrocatechol, p-coumaric acid, and ferulic acid, respectively.

near the solvent front as a streak, i.e., R_f = 0.85-0.95. No further work was done with this fraction.

THE HEXANE-SOLUBLE MATERIALS

The materials found in this fraction were waxy and slightly yellow in color. These materials moved with the solvent front in TAW and gave the characteristic pink color with the Wiesner test reagent, which is probably due to oxidized fatty materials (78). Attempts to resolve these materials with BAW resulted in considerable streaking on the chromatograms but no apparent resolution of spots. The Wiesner-positive materials and those detected in the ether-neutral fraction were chromatographically similar.

THE PRECIPITATES-FROM-WATER

The precipitates-from-water were dark brown viscous oils which were readily soluble in methanol. These materials were chromatographed with BAW and TAW. When employing TAW, these materials moved quite slowly and always streaked from the origin. However, partial resolution was accomplished with BAW, although considerable streaking was also encountered. The chromatographic properties of the precipitates-from-water were reminiscent of similar materials isolated from the xylem (63). Chromatographic data concerning these materials are shown in Tables XIV and XV.

THE PRECIPITATES-FROM-ETHER

Orienting paper chromatography of these materials with BAW and TAW revealed that these materials had movement characteristics similar to the precipitates-from-water, i.e., with BAW, the materials moved with the solvent front with streaking and with TAW, part of the materials streaked from the origin but the major portion did not move.

TABLE XIV

CHROMATOGRAPHIC PROPERTIES OF THE INNER PHLOEM PRECIPITATES-FROM-WATER

Developer: BAW

Spray		Sample T-74,75	T-76,77	T-81,82	T-88,89,90
Mäule	R_f	-	-	0.81(weak)[a]	0.81(weak)
Wiesner		-	0.85	0.87	0.69-0.94(streak)
FC		(not determined)	0.61 0.85	0.67-0.83(streak)	0.53-0.86(streak)
DNPH		0.80	0.57-0.66(streak) 0.79	0.69 0.86	0.57-0.85(streak)
PNA		0-0.83(streak) 0.49 0.68 0.77 0.83	0-0.83(streak) 0.63 0.69 0.83	0-0.85(streak) 0.61 0.69 0.77 0.85	0-0.83(streak) 0.62 0.70 0.83

[a]The Mäule-positive materials are quite dilute considering the amount of material spotted on the papers.

TABLE XV

CHROMATOGRAPHIC PROPERTIES OF THE STONE CELL LAYER PRECIPITATES-FROM-WATER

Developer: BAW

Spray		Sample T-74,75	T-76,77	T-81,82	T-88,89,90
Mäule	R_f	-	-	0.59(weak)[a] 0.77(weak)	0.55(weak)
Wiesner		-	0.79	0.82	0.71-0.84(streak)
FC		(not determined)	0.53 0.61-0.75(streak)	0.66-0.83(streak)	0.57-0.80(streak)
DNPH		0.80	0.48-0.84(streak)	0.69 0.83	0.63 0.74-0.87(streak)
PNA		0-0.83(streak) 0.24 0.32 0.49 0.68 0.77 0.83	0-0.75(streak) 0.61 0.67 0.84	0-0.83(streak) 0.59 0.66 0.75 0.83	0-0.83(streak) 0.64 0.72 0.83

[a]The Mäule-positive materials are quite dilute considering the amount of material spotted on the papers.

SEASONAL VARIATION

A secondary objective of this study was to determine whether there were any qualitative differences between methanol extracts collected at various times throughout a one-year growing cycle. Paper chromatography was employed for this comparison. It should be noted that the samples collected do not represent enough trees to make any statistically valid conclusions. The data presented, however, serve to give a general idea about the behavior of these extracts.

Tables XVI and XVII summarize these data for the major fractions of these extracts.

TABLE XVI

SEASONAL VARIATION OF THE MAJOR FRACTIONS OF THE INNER PHLOEM METHANOL EXTRACTS

Fraction	Color Test or Compound[a]	Season: Fall	Winter	Spring	Summer
Water-soluble materials (hexane and ether extracted)	Mäule	+(weak-streak)[b]	+(weak-streak)	+(weak-streak)	+(weak-streak)
	Wiesner	–	+(streak)	+(streak)	+
	sucrose	+	+	+	+
	glucose	+	+	+	+
	fructose	+	+	+	+
	salicin	+	+	+	+
	populin	+	+	+	+
	tremuloidin	+	+	+	+
	salireposide	+	+	+	+
Precipitates-from-water	Mäule	–	–	+(weak)	+(weak)
	Wiesner	–	+(one spot)	+(one spot)	+(streak)
	FC	(not determined)	+(2 spots)	+(streak)	+(streak)
	PNA	+(5 spots)	+(3 spots)	+(streak)	+(streak)
	DNPH	+(2 spots)	+(streak)	+(streak)	+(streak)
Ether-soluble materials (except ether neutrals)	Mäule	–	–	–	–
	Wiesner	–	+(one spot)	+(one spot)	+(2 spots)
	p-hydroxy-benzoic acid	+	+	+	+
	p-coumaric acid	+	+	+	+
	ferulic acid	–	+	+	+
	vanillic acid	+	–	–	–
	pyrocatechol	+	+	+	+

TABLE XVI (continued)

SEASONAL VARIATION OF THE MAJOR FRACTIONS OF THE INNER PHLOEM METHANOL EXTRACTS

Fraction	Color Test or Compound[a]	Season: Fall	Winter	Spring	Summer
Hexane-soluble materials	Mäule	-	-	-	-
	Wiesner	+(one spot)	+(one spot)	+(one spot)	+(one spot)
	FC	-	-	-	-
	PNA	-	-	-	-
	DNPH	+(one spot)	+(one spot)	+(one spot)	+(one spot)
Ether neutrals	Mäule	-	-	-	-
	Wiesner	+(streak)	+(streak)	+(streak)	+(streak)
	FC	-	-	-	-
	PNA	-	-	-	-
	DNPH	+(streak)	+(streak)	+(streak)	+(streak)

[a]All results are based on paper chromatography. At least two developing solvent systems were used.

[b]The Mäule-positive materials are quite dilute considering the amount of material spotted on the papers.

TABLE XVII

SEASONAL VARIATION OF THE MAJOR FRACTIONS OF THE STONE CELL LAYER METHANOL EXTRACTS

Fraction	Color Test or Compound[a]	Season			
		Fall	Winter	Spring	Summer
Water-soluble materials (hexane and ether extracted)	Mäule	+[b]	+(weak-streak)	+(weak-streak)	+(weak-streak)
	Wiesner	+(one spot)	+(weak-streak)	+(weak-streak)	-
	sucrose	+	+	+	+
	glucose	+	+	+	+
	fructose	+	+	+	+
	salicin	(not determined)	+	+	+
	populin	(not determined)	+	+	+
	tremuloidin	(not determined)	+	+	+
	salireposide	(not determined)	+	+	+
Precipitates-from-water	Mäule	-	-	+(weak)	+(weak)
	Wiesner	-	+(one spot)	+(one spot)	+(streak)
	FC	(not determined)	+(streak)	+(streak)	+(streak)
	PNA	+(7 spots)	+(3 spots)	+(streak)	+(streak)
	DNPH	+(2 spots)	+(streak)	+(streak)	+(streak)
Ether-soluble materials (except ether neutrals)	Mäule	-	-	-	-
	Wiesner	-	+(one spot)	+(one spot)	+(one spot)
	p-hydroxy-benzoic acid	+	+	+	+
	p-coumaric acid	+	+	+	+
	ferulic acid	-	+	+	+
	vanillic acid	-	-	+	-
	pyrocatechol	+	+	+	+

TABLE XVII (continued)

SEASONAL VARIATION OF THE MAJOR FRACTIONS OF THE STONE CELL LAYER METHANOL EXTRACTS

Fraction	Color Test or Compound[a]	Season: Fall	Winter	Spring	Summer
Hexane-soluble materials	Maüle	-	-	-	-
	Wiesner	+(one spot)	+(one spot)	+(one spot)	+(one spot)
	FC	-	-	-	-
	PNA	-	-	-	-
	DNPH	+(one spot)	+(one spot)	+(one spot)	+(one spot)
Ether neutrals	Maüle	-	-	-	-
	Wiesner	+(streak)	+(streak)	+(streak)	+(streak)
	FC	-	-	-	-
	PNA	-	-	-	-
	DNPH	+(streak)	+(streak)	+(streak)	+(streak)

[a] All results are based on paper chromatography. At least two developing solvent systems were used.

[b] The Maüle-positive materials are quite dilute considering the amount of material spotted on the papers.

DISCUSSION

Of the various hypotheses concerning the formation of lignin, the one proposed by Klason and later extended by Freudenberg is the basis of this thesis (8-10). During the course of this study, ten aromatic compounds have been identified. The fact that certain of these compounds are in harmony with the biosynthetic scheme is not proof that they are lignin precursors. However, the absence of expected compounds and the presence of other aromatic materials may alter our ideas concerning the biological formation of lignin in the particular species P. tremuloides. What the presence of these compounds means will be examined primarily in the light of the newer views of lignin formation. Since the compounds identified in this study can be classified according to their structure, the discussion to follow is divided into sections based on their classification and on the postulated biosynthetic pathway. When discussing the nature of the data obtained in this study, the views of the organic chemist are best exemplified by A. J. Birch's statement:

> The organic chemist assumes that reactions which take place in living matter, whether enzyme-catalyzed or not, take place by mechanisms which are explicable in terms of the electronic theory of reaction mechanisms which has been elaborated in the laboratory. This is not to deny that enzymes may possess a specificity in terms particularly of stereochemistry which can be imitated only with difficulty or not at all in the laboratory, and that many energy-consuming processes may occur with astonishing ease. The organic chemist is not happy, however, in postulating a process in living matter which has no laboratory analogy or for which a satisfactory mechanism cannot be proposed. If there is no known analogy his instinct is to provide one. In assessing the feasibility of a possible biochemical reaction the emphasis is always therefore on: (1) whether a possible mechanism clearly exists; and (2) whether such a mechanism could conceivably operate, or is known to operate, in biochemical reactions (90).

GLUCOSE, FRUCTOSE, SUCROSE

Since the simple sugars are the major products of photosynthesis (15,16), they must be considered early precursors to the aromatic materials found in woody plants. The biological function of these sugars, with respect to the synthesis of lignin, can be briefly summarized. Glucose and fructose constitute a hexose pool from which sedoheptulose derivatives are synthesized. Through cyclization, and a series of oxidation and reduction reactions, these 7-carbon atom sugars yield the hydroaromatic acids which are converted by several steps into the aromatic amino acids and/or the aromatic lignin building stones (Figures 3, 4, 5 and 8). Sucrose serves as a storage for the two simple sugars and as a supply of glucose for the synthesis of cellulose.

PARA-COUMARIC ACID AND FERULIC ACID

Para-coumaric acid and ferulic acid, two aromatic C_6-C_3 compounds considered to be precursors to coniferyl alcohol, were detected in the inner phloem of aspen. In addition, it has been shown that an alkaline hydrolysis of whole aspenwood yielded small amounts of p-coumaric acid but no ferulic acid (91). The same results were obtained with a methanol extract of newly formed aspenwood (92). Thus, p-coumaric acid has been found in both the xylem and inner phloem of aspen, but ferulic acid has been detected only in the phloem tissue.

XYLEM LIGNIN

The lignin of aspenwood is Mäule-positive, indicating the presence of syringyl groups. If the Klason-Freudenberg hypothesis is valid, the presence of low-molecular weight syringyl derivatives would be expected in lignifying xylem tissues. And indeed, at least four ether-soluble, Mäule-positive materials, three of which have been identified as syringaldehyde, syringic acid and sinapaldehyde, have been detected in various aspenwood tissues (63, 65, 74). However, in the inner phloem, all of the ether-soluble materials extracted by methanol were Mäule-negative.

Angiosperm dicotyledonous lignin is thought to be primarily composed of guaiacyl and syringyl nuclei. The data of this study showed that inner phloem ether-soluble materials were Mäule-negative. If some of the aromatic materials of this tissue do indeed move into the xylem, the questions arise: Where does the syringyl nucleus originate? Is it formed from the guaiacyl or the p-hydroxyphenyl nuclei found in the inner phloem? If p-coumaric acid and ferulic acid, which are present in the inner phloem, are considered lignin precursors and consequently are transported into the xylem, it appears that the cambium may affect in some manner the synthesis of compounds having the syringyl nucleus. Ferulic acid apparently is not a source of syringyl nuclei since the guaiacyl nucleus, once formed, supposedly does not undergo further methoxylation during lignification (52). Thus, the p-hydroxyphenyl nucleus of p-coumaric acid may be transformed into the syringyl nucleus to some degree during its transport through the cambium. If p-coumaric acid were undergoing methoxylation in the cambial zone, small quantities

of the p-hydroxyphenyl nucleus could possibly be incorporated into the lignin. This event would probably explain the detection of this acid by an alkaline hydrolysis of xylem.

On the other hand, the absence of ferulic acid in the methanol extracts (63) and in the alkaline hydrolyzates of the methanol extracts of aspenwood (92) is paradoxical. If ferulic acid and p-coumaric acid are lignin precursors, the absence of ferulic acid in aspen xylem suggests either (1) this acid may be completely incorporated into the lignin so that none is available for extraction or hydrolysis, or (2) ferulic acid may be altered when transported from the phloem into the xylem.

PHLOEM LIGNIN

This study revealed that the low-molecular weight, ether-soluble extractives of inner phloem and stone cell layer were Mäule-negative. Conversely, the methanol-extracted inner phloem and stone cell layer tissues which contain high-molecular weight materials, i.e., methanol-insoluble, were Mäule-positive. These data suggest that the biosynthetic pathways to phloem lignin differ from those proposed for xylem lignin. If some of the aromatic materials of these tissues serve as phloem lignin precursors, the question again arises: Where does the syringyl nucleus, present in the high-molecular weight materials of phloem, originate? If p-coumaric acid and ferulic acid are considered precursors to the phloem lignin, the pathways leading to the formation of syringyl nuclei in the phloem from either of these acids are apparently not parallel to the pathways mentioned above for the formation of syringyl nuclei in xylem.

The possibility of a direct polymerization of p-coumaric acid and ferulic acid, or their corresponding alcohols, followed by some hydroxylation and/or methoxylation would be in harmony with the above evidence. However, in a discussion of the possibility of rather specific reactions occurring in a preformed polymer, i.e., the reduction of terminal side chain carboxyl groups in lignin, it has been stated (5): "---that such enzymatic transformations in a preformed high-molecular material of the type of lignin appear to have little probability."

The Precipitates-from-Water

The phloem precipitates-from-water are dark brown viscous oils whose chromatographic behaviors are reminiscent of xylem-isolated lignin (63). Since these precipitates are methanol-soluble and water-insoluble, they probably contain some higher molecular weight materials. It should be noted that these precipitates were Mäule-negative when the cambium was dormant, but were Mäule-positive during the spring and summer months. Assuming the seasonal variation data are valid, it is suggested that hydroxylation and/or methoxylation in the bark tissues may occur when the cambium is active.

THE GLUCOSIDES -- SALICIN, POPULIN TREMULOIDIN, SALIREPOSIDE

The current views of lignin formation postulate the presence of the glucosides coniferin and syringin in angiosperm dicotyledons (Figure 8). The actual isolation or detection of these glucosides, however, is limited to comparatively few species of woody plants (93), and they have not been

detected in this study. But the evidence presented in this thesis and elsewhere (88,89) reveals the presence of four other glucosides in aspen inner bark, salicin, populin, tremuloidin and salireposide.

If glucoside-hydrolyzing enzymes are present in the cambial sap of aspen, as has been shown in the case of spruce (10), these saligenin glucosides might be hydrolyzed if they move into and through the cambial zone. Recently, salicin has been isolated from the soft xylem of aspen (94), suggesting some inward radial movement. The basic aromatic C_6-C_1 unit with the ortho-hydroxyl substitution has not been found in mature xylem, although there is evidence for a structure of the aromatic C_6-C_1 type with a parahydroxyl substitution being a part of the cell wall of aspen xylem (74).

It is interesting to note that saligenin is the first reaction product of the well-known phenol-formaldehyde condensation reaction.

Whether this type of reaction may be occurring in the cambial layer or phloem yielding ligninlike polymers of relatively low methoxyl substitution can only be speculative. But it should be noted that the reactions as well as the reactivities of saligenin, coniferyl alcohol and sinapyl alcohol are quite similar in dilute acid, all three compounds yielding amorphous resins (95). The alkaline and the acidic instability of coniferyl alcohol was emphasized during the isolation of this compound from Siam

benzoin (96). The similar reactivities of these alcohols would be expected since the only essential difference between coniferyl and sinapyl alcohols and saligenin is the presence of an allylic system. Thus, if glucoside-hydrolyzing enzymes are present in the cambial sap of aspen, one might expect the cleavage of the glucosides to yield the highly reactive alcohols which, under the mildly acidic conditions of the sap, could polymerize. The lignin of aspen, therefore, could possibly contain some units of saligenin in addition to coniferyl and sinapyl alcohols.

PARA-HYDROXYBENZOIC ACID, VANILLIC ACID, BENZOIC ACID

The current views of lignin biosynthesis do not postulate the presence of either vanillic, benzoic or p-hydroxybenzoic acids (Figure 8). However, it has been shown that p-hydroxybenzoic acid is a necessary growth factor for several micro-organisms which were employed in the studies of aromatic amino acid biosynthesis (21) (Figure 5). It has been suggested that the biosynthetic pathways leading to the synthesis of aromatic amino acids in these micro-organisms may have general applicability to higher plants (5), and thus, this acid may be serving, in part, as a growth factor in P. tremuloides.

Extensive studies on hardwoods have shown that p-hydroxybenzoic acid was present in relatively large quantities only in the closely related Populus and Salix genera (97). This acid, however, has also been found in several gymnosperms, tropical hardwoods and other angiosperm dicotyledons (98). It has been demonstrated, in the case of aspenwood, that this acid is not only an extractive (63), but also part

of the cell wall (74), a constituent of "isolated native aspen lignin" (99), and associated with the Klason lignin of aspenwood (100). This acid also occurs as an ester in aspenwood lignin (98). The close association of p-hydroxybenzoic acid with aspenwood lignin suggests that this acid may be considered to some degree a plasticizer of this lignin system.

The presence of vanillic and p-hydroxybenzoic acids in the inner phloem of aspen could result from the oxidation of ferulic acid and p-coumaric acid, respectively. A possible relationship between p-coumaric acid and p-hydroxybenzoic acid in aspen was borne out to some extent by the alkaline hydrolyses of the water-soluble materials of the methanol extracts of whole inner bark, soft- and year-old xylem (92). The concentrations of p-coumaric acid relative to p-hydroxybenzoic acid were noted to be: whole inner bark, 8/1; soft xylem, 5/1; and year-old xylem, 1/5. The depletion of p-coumaric acid and the corresponding increase of p-hydroxybenzoic acid going from phloem to xylem suggests that these acids may be related.

Populin, tremuloidin and salireposide contain the benzoate group. The presence of benzoic acid in the inner phloem could theoretically be attributed to a mild hydrolysis of these glucosides. The rather large reserve of benzoic acid in these glucosides could also account for the relatively large amounts of p-hydroxybenzoic acid present in aspenwood through a hydroxylation mechanism previously postulated to be influenced by the cambium.

Although several ideas have been suggested above to explain the presence of these acids both in inner phloem and xylem tissues, and their possible relationships to lignification, the fact that p-hydroxybenzoic acid is so intimately associated with aspenwood lignin is notably different from the hypothesis presented concerning the structure of hardwood lignins.

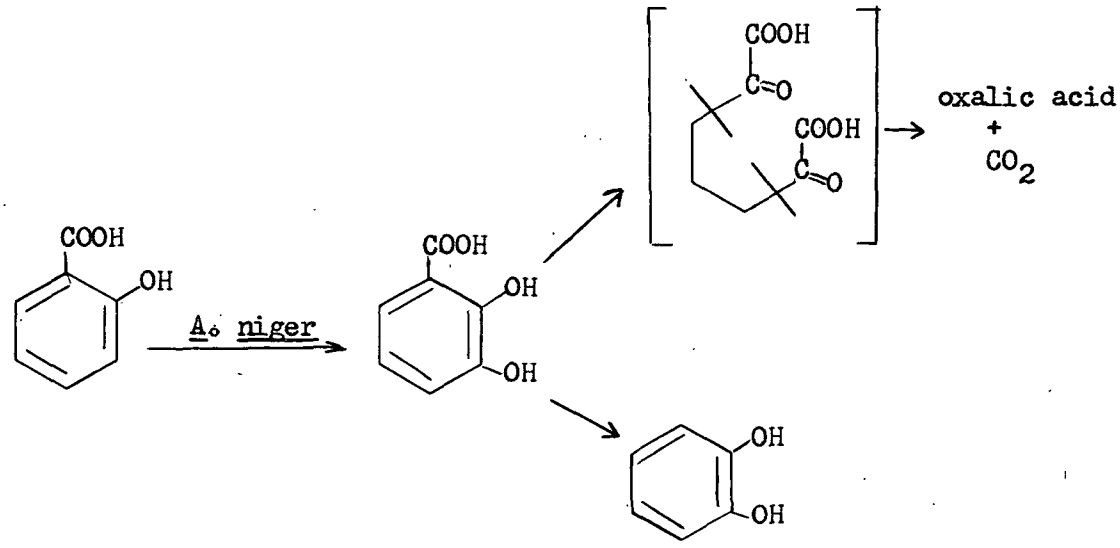
PYROCATECHOL

Pyrocatechol has been shown to occur in beets (101), sycamore bark (102), onion skins (103), potato tubers (104), grapes (105), Sequoia cone solids (106), and in the roots and bark of numerous other woody plants (107-109).

Several authors have speculated that pyrocatechol may have an influence upon the disease resistance of the plant in question (103,106). Since P. tremuloides is known to be quite susceptible to disease (110), it would be interesting to determine if pyrocatechol had any such biological function in this instance.

It is also interesting to discuss the possible origin of this relatively simple phenol. A study of the metabolism of salicylic acid by A. niger revealed that pyrocatechol and oxalic acid were the end products (111). The following reactions were proposed and were based on the isolation of the intermediate 2,3-dihydroxybenzoic acid.

The presence of salicin in the bark of P. tremuloides could be considered a source of salicylic acid through a hydrolysis and mild



oxidation reaction scheme. Consequently, if the proper enzyme systems are available, the presence of pyrocatechol in aspen bark would be expected.

It should also be noted in this respect that several compounds have been isolated from fungal cultures which were not considered part of the direct pathway from glucose to shikimic acid but were considered to be accumulated as a result of a "bottleneck" in the direct pathway (31) (Figure 4). These compounds were quinic acid, protocatechuic acid and pyrocatechol. Thus, it was concluded that these secondary compounds can be accumulated depending on the equilibria involved in the particular species of organism.

While neither of the two pathways discussed above have been shown to exist in P. tremuloides, most of the evidence leads one to hypothesize that pyrocatechol is probably a metabolic end product in aspen bark.

CONCLUSIONS

The following conclusions are based on the data obtained in this study and other relevant information:

1. Some of the compounds identified are in harmony with certain points of the postulated biosynthetic pathways to lignin, i.e., the sugars and the phenolic C_6-C_3 carboxylic acids, but they do not exactly match the compounds detected in aspen xylem, i.e., sinapaldehyde and the aromatic amino acids (63).

2. Glucose, sucrose and fructose, present in the inner phloem and stone cell layer of aspen bark, constitute a carbohydrate reserve which is comparable to aspen xylem and from which the lignin building stones may be synthesized.

3. Of the aromatic C_6-C_3 compounds postulated in the pathways to lignin, p-coumaric acid and ferulic acid were present in the free state and represent an earlier stage in the pathways than the sinapaldehyde present in aspen xylem.

4. Although p-hydroxybenzoic acid and pyrocatechol are not included in the direct pathways to lignin, they may still be regarded as metabolic side products of them.

5. The occurrence of syringin and coniferin in aspen bark was anticipated but not established; instead, four glucosides, derived from saligenin, were present, i.e., salicin, populin, tremuloidin and salireposide.

6. The fact that p-coumaryl, coniferyl and sinapyl alcohols are vinylogues of the p-isomer of saligenin, the first product of the phenol-formaldehyde condensation reaction, suggests analogies between the chemistry of phenol-formaldehyde resins and lignin.

7. Until more information is obtained concerning the role of benzoic acid in aspen inner phloem, the simplest explanation of its occurrence is that it is associated with the aforementioned glucosides, except salicin.

8. The sporadic occurrence of vanillic acid in the inner bark of aspen does not warrant any present attempt to relate it to the postulated pathways to lignin.

9. The available data indicate a difference in the development of Mäule-positive materials in xylem as compared to phloem in that sinapaldehyde and similar compounds were present in lignifying xylem, but were absent in the inner bark.

10. In the case of nine aspen barks studied, Mäule-positive materials found in the precipitates-from-water were present only during the growing season, suggesting a possible association between the formation of Mäule-positive materials and the activity of the cambium.

11. Although the compounds, shikimic acid, cinnamic acid, p-coumaraldehyde, coniferaldehyde, sinapaldehyde, coniferin and syringin have been regarded as important steps on the pathways to lignin, they were not detected in any of the inner phloem or stone cell layer

extracts; but these findings do not preclude the possibility that these compounds may be transitory intermediates.

12. The apparent uniformity of structure of aspen inner phloem as compared to the structural heterogeneity of the stone cell layer is paralleled to some degree by their chemical complexity as shown by chromatographic analysis of the water-soluble materials and the precipitates-from-water.

13. The monitoring of the fractionation procedures by infrared spectral analysis may be a useful addition to qualitative organic analyses.

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The various definitions given for lignin were presented. Lignin was first described as the encrusting material of woody plants.

2. Björkman, A., and Person, B., Svensk Papperstidn. 60:158-69(1957).

Milled wood lignin was prepared from several wood species and compared analytically. The isolated lignins from Picea mariana, Pinus sylvestris, Tsuga heterophylla, Thuja plicata, and Picea abies were very similar with respect to composition and absorption of ultraviolet light. The isolated lignins from Betula verrucosa, Populus tremula, and Fagus silvatica showed much larger differences than the gymnosperm lignins. The $\Delta\epsilon$ curves of lignin preparations from P. tremula were shown to vary within the same tree.

3. Sharkov, V. I., Efimov, V. A., Muromtseva, V. S., and Tsalikova, A. V., Zhur. Priklad. Khim. 26:626-39(1953); C.A. 48:9686.

Five disks of wood from P. sylvestris and P. excelsa were obtained from varying heights in the trees. Each of the disks were then subdivided into five groups according to the age of the wood. Extensive analyses were then made on all of these wood samples. The % lignin in the pine varied from 26.2 to 28.7 and in spruce from 27.0 to 28.9. The % methoxyl varied from 13.0 to 14.3 in the pine lignin and from 13.8 to 16.8 in the spruce lignin. There was no correlation between these values and the location of the sample within the tree.

4. Brauns, F. E. The chemistry of lignin. 1st ed. New York, Academic Press Inc., 1952. 808 p.

A complete review of the chemistry of lignin through 1950 was presented.

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The newer views of lignin formation were summarized and critically analyzed in the light of recent experimental work by the following authors: Adler, Björkman, Freudenberg, Tatum, Davis, Ehrensvärd, Brown, Neish, Eberhardt, Schubert, Nord, Udenfriend, Mason, Byerrum, Siegel, Erdtman and others. The biosynthetic pathways to lignification involved the synthesis from glucose of the aromatic amino acids, tyrosine and phenylalanine, via the hydroaromatic acids, dehydroquinic, dehydroshikimic, shikimic and prephenic. The hydroxylation and methoxylation of the aromatic nucleus and the polymerization into lignin of the hypothesized phenylpropane building stones were discussed in detail. The structural elements thought to be present in the gymnosperm lignin system were described.

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The consensus of opinion concerning the translocation of materials within the tree was briefly summarized. Materials elaborated in the leaves move down the tree in the inner bark. Mineral salts in very dilute solution and reserve food that has been rendered soluble after the winter, travel upward in the sapwood.

8. Klason, P., Svensk Kem. Tidskr. 9:133(1897); cf. F. E. Brauns (4).

The first theory concerning the formation of lignin was proposed and included a possible structural formula of an aromatic nature.

9. Klason, P., Svensk Kem. Tidskr. 9:135(1897); cf. F. E. Brauns (4).

The first working hypothesis that lignin is in some manner related to coniferyl alcohol was proposed.

10. Freudenberg, K., Reznik, H., Boesenberg, H., and Rasenack, D., Chem. Ber. 85:641-7(1952).

A strictly localized beta-glucosidase was shown to be present in the cambial zone of conifers and several hardwoods. The dehydrogenation and oxidation enzymes extracted from this zone formed a dehydrogenation polymer (DHP) from coniferyl alcohol. A theory of lignification based on this evidence was proposed. Essentially, coniferyl alcohol is released from coniferin by the localized beta-glucosidase. The alcohol is then polymerized to lignin with the aid of phenol dehydrogenase and peroxidase. The authors continued:

"This conception presupposes that the coniferin and syringin are led from the still unknown places of formation to the lignifying tissues. It might also be supposed that these hydroxycinnamic alcohols are synthesized at the places of lignification and that the glucosidases serve to fix the excess by conversion to glucosides. This explanation is less probable than the first because the hydroxycinnamic alcohols would have to be produced at different points in the tissues in which such biosynthetic capacities probably do not exist."

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The early work done in elucidating the various photosynthetic steps was reviewed. The use of radioactive carbon and various methods of analysis were described. In studies with green algae, fifteen compounds have been identified after 90 seconds of photosynthesis. 2-Phosphoglyceric acid was one of the first formed compounds.

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The immediate precursor to shikimic acid was isolated with E. coli mutant 83-2. This compound has been tentatively identified as 5-dehydroshikimic acid.

18. Weiss, U., Davis, B. D., and Mingioli, E. S., J. Am. Chem. Soc. 75:5572-6(1953).

5-Dehydroquinic acid has been identified as a precursor to 5-dehydroshikimic acid and shikimic acid. E. coli mutant 170-27 was employed for this work.

19. Weiss, U., Gilvarg, C., Mingioli, E. S., and Davis, B. D., Science 119:774-5(1954).

Prephenic acid was isolated from a culture of E. coli mutant 83-5. It was considered an intermediate between shikimic acid and phenylpyruvic acid.

20. Yaniv, H., and Gilvarg, C., J. Biol. Chem. 213:787-95(1955).

The enzyme which was responsible for the reduction of 5-dehydroshikimic acid to shikimic acid was isolated from E. coli and partially purified.

21. Davis, B. D. In McElroy and Glass's Symposium on amino acid metabolism. p. 799-811. Baltimore, Johns Hopkins Press, 1955.

The biosynthetic pathway from dehydroquinic acid to the aromatic amino acids was presented. Also discussed were the preparation of the mutant bacteria and the necessary aromatic growth factors, tyrosine, phenylalanine, tryptophan, p-aminobenzoic acid, p-hydroxybenzoic acid and a sixth unknown factor.

22. Gilvarg, C. In McElroy and Glass's Symposium on amino acid metabolism. p. 812-6. Baltimore, Johns Hopkins Press, 1955.

Culture filtrates of E. coli mutant 83-5 were used for the isolation of prephenic acid. Since it was determined that this

acid contained ten carbon atoms and that the carboxyl carbon atom of shikimic acid was lost in its conversion to phenylalanine, it was concluded that prephenic acid was an intermediate in which both the side chain and the carboxyl group coexisted attached to the ring.

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Radioactive glucose, specifically labeled in the 1,2,3, and 6 positions were used in biosynthetic studies of shikimic acid. E. coli mutant 83-24 was used in this work. The synthesized shikimic acid was analyzed for radioactivity in each position. From the variation of radioactivity within the shikimic acid molecule, it was evident that glucose did not remain intact during its incorporation into this acid, and it was concluded that an unknown path of glucose metabolism was involved.

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Sedoheptulose-1,7-diphosphate was shown to be efficiently converted to 5-dehydroshikimic acid by E. coli mutant 83-2.

25. Srinivasan, P. R., Katagiri, M., and Sprinson, D. B., J. Am. Chem. Soc. 77:4943-4(1955).

Evidence was presented which indicated that sedoheptulose-1, 7-diphosphate was broken down into erythrose-4-phosphate and phosphenol pyruvate. The recondensation of these two intermediates yielded 2-keto-3-deoxy-7-phospho-D-glucoheptonic acid, in which carbons 4 and 5 had the same configuration as dehydroshikimic acid.

These studies were made with a cell-free extract of E. coli mutant 83-24 which had been subjected to ultrasonic vibrations.

26. Srinivasan, P. R., Shigeura, H. T., Sprecher, M., Sprinson, D. B., and Davis, B. D., J. Biol. Chem. 220:477-97(1956).

E. coli mutant 83-24 which accumulates shikimic acid was employed to test variously labeled C^{14} glucoses, unlabeled glucose and labeled bicarbonate, formate, acetate and pyruvate. The latter compounds were not incorporated into shikimic acid; however, it was found that the carboxyl, C-1 and C-2 fragment of shikimic acid was derived from a 3-carbon intermediate of glycolysis and the C-3,4,5,6 fragment from tetrose phosphate via the pentose phosphate pathway. The orientation of the two fragments seemed incompatible with the formation of shikimic acid through cyclization of the intact chain of heptose. When 6-C^{14}-D-glucose was used, the shikimic acid contained 52% of its total activity in position 6 and 43% in position 2.

27. Kalan, E. B., Davis, B. D., Srinivasan, P. R., and Sprinson, D. B., J. Biol. Chem. 223:907-12(1956).

All of the uniformly labeled sugars tested, except 6-phosphogluconic acid, were converted to dehydroshikimic acid by E. coli mutant 83-2, which was blocked after dehydroshikimic acid. These sugars were glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate and sedoheptulose-7-phosphate.

28. Srinivasan, P. R., Sprinson, D. B., Kalan, E. B., and Davis, B. D., J. Biol. Chem. 223:913-20(1956).

Sedoheptulose-1,7-diphosphate, labeled in positions 4,5,6, and 7, was incorporated into shikimic acid by a mutant of E. coli. Furthermore, the isolated shikimic acid was labeled exclusively in positions 3,4,5, and 6 without dilution. The authors concluded that the carbon atoms 3,4,5, and 6 of shikimic acid arose from tetrose phosphate and the other three carbon atoms from triose phosphate. From previous studies with glucose, it was concluded that the formation of shikimic acid could not involve cyclization of the intact chain of sedoheptulose.

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Shikimic acid 5-phosphate has been isolated and identified in a culture of Aerobacter aerogenes strain A170-40. It has been shown by E. B. Kalan that this compound is a true intermediate between shikimic acid and prephenic acid; cf. footnote 9a, this article.

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The relationship between shikimic acid and 5-dehydroshikimic acid were traced with Neurospora mutant 7655. The block in the mutant was between these two acids. In Neurospora, 5-dehydroshikimic acid was not accumulated, but was rapidly converted to protocatechuic acid.

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The occurrence of shikimic acid in 49 species of higher plants was reported.

33. Hattori, S., Yoshida, S., and Hasegawa, M., Physiol. Plant 7:283-9 (1954).

Shikimic acid was found in the leaves of 30 gymnosperms of 34 tested. The gymnosperm families tested included Cupressaceae, Taxodiacea, Pinaceae, Taxaceae, Cephalotaxaceae, Podocarpaceae, Araucariaceae, Cycadaceae and Ginkgoceae.

34. Anet, E. F. J. L., Birch, L. S., and Massey-Westropp, R. A., Australian J. Chem. 10:93-4(1957).

Shikimic acid was found in the young leaves of Eucalyptus citriodora.

35. Whiting, G. C., Nature 179:531(1957).

Shikimic acid was found in the fruits of gooseberry plants. This acid was also found in the carpels of Illicium verum, in blackberries, cider apples, perry pears, quince fruits and the grass Lolium perenne.

36. Fieser, L. F., and Fieser, N. Organic chemistry. 2d ed. p. 535-49. Boston, D.C. Heath and Co., 1950.

The metabolism of proteins and amino acids was reviewed. The interconversion of carbonyl groups and amino groups occurs quite readily in animal and plant metabolism.

37. Eberhardt, G., and Nord, F. F., Arch. Biochem. Biophys. 55:578-9 (1955).

Lentinus lepideus was grown on glucose. The resulting growth medium contained oxaloacetic acid, alpha-keto-glutaric acid, pyruvic acid, acetoacetic acid, p-hydroxyphenylpyruvic acid and a precipitate of methyl p-methoxycinnamate. It was concluded that p-hydroxyphenylpyruvic acid was a direct precursor of methyl p-methoxycinnamate and that the citric acid cycle was probably operative in this instance.

38. Eberhardt, G., J. Am. Chem. Soc. 78:2832-5(1956).

On the basis of biosynthetic studies with methyl p-methoxy-cinnamate, it was concluded that p-hydroxycinnamyl alcohol should be considered a lignin building stone in certain plants. The relationship between p-hydroxycinnamic acid an tyrosine was discussed.

39. Schubert, W. J., Acerbo, S. N., and Nord, F. F., J. Am. Chem. Soc. 79:251-2(1957).

Radioactive p-hydroxyphenylpyruvic acid, $-C^{14}OOH$, was prepared and fed to sugar-cane plants. The lignin, isolated after 15 days of metabolism, was not only radioactive but contained at least 70% of the administered C^{14}. It was concluded that p-hydroxy-phenylpyruvic acid was an intermediate between shikimic acid and the lignin building stones.

40. Nord, F. F., and Schubert, W. J., Tappi 40:285-94(1957).

During the isolation of lignins employing the brown rot fungi, it was discovered that Lentinus lepideus produced methyl p-methoxy-

cinnamate as a terminal product when grown on carbohydrates or ethanol. Intermediate metabolites found in these cultures were sedoheptulose, shikimic acid and p-hydroxyphenylpyruvic acid. It was concluded that p-hydroxyphenylpyruvic acid was an intermediate between shikimic acid and tyrosine and could also be a precursor to the lignin building stone p-hydroxycinnamyl alcohol.

41. Acerbo, S. N., Schubert, W. J., and Nord, F. F., J. Am. Chem. Soc. 80:1990(1958).

Carboxyl-labeled p-hydroxyphenylpyruvic acid was fed to sugar-cane plants, and a portion of the resulting lignin was oxidized with nitrobenzene. The vanillin obtained was not radioactive. Alkaline fusion of the lignin yielded radioactive oxalic acid. Therefore, it was suggested that the aromatic ring of the acid was converted into the aromatic rings in lignin, without randomization, and that the side chain of p-hydroxyphenylpyruvic acid was not involved in the aromatization process.

42. Shimazono, H., Schubert, W. J., and Nord, F. F., J. Am. Chem. Soc. 80:1992-4(1958).

Lentinus lepideus was grown on a culture medium containing 1-C^{14}-D-glucose and 6-C^{14}-D-glucose. The activities of both sugars were significantly incorporated into the metabolic product methyl p-methoxycinnamate. The comparative distribution of radioactivity in the ester from 6-C^{14}-D-glucose suggested that the ester was formed via shikimic acid and prephenic acid.

43. Udenfriend, S., and Mitoma, C. In McElroy and Glass's Symposium on amino acid metabolism, p. 876-81. Baltimore, Johns Hopkins Press, 1955.

Oxygen, simple aldehydes (except formaldehyde) and ferrous ions have been used with hydroxylating enzymes found in animal liver to convert phenylalanine to tyrosine. These enzymes were specific for L-phenylalanine.

44. Mason, H. S., Fowlks, W. L., and Peterson, E., J. Am. Chem. Soc. 77:2914-15(1955).

Tests on the introduction of hydroxyl groups into the benzene nucleus revealed that with the "phenolase" complex, all of the hydroxyl groups were derived from molecular oxygen and not water. The "phenolase" complex was thus an oxygen transferase.

45. Byerrum, R. V., Flockstra, J. H., Dewey, L. J., and Ball, C. D., J. Biol. Chem. 210:633-43(1954).

It was demonstrated that the methyl group of methionine could take part in transmethylation reactions in higher plants, specifically in tobacco and barley. It was also found that this methyl group appeared in the methoxyl groupings of the isolated lignin from these plants. C^{14}-formate was also shown to be a precursor of the methoxyl groups in lignin but was only 1/26 as effective as methionine.

46. Hamill, R. L., Byerrum, R. V., and Ball, C. D., J. Biol. Chem. 224:713-16(1957).

About 90% of the labeled carbon atoms of glycine-2-C^{14}, serine-3-C^{14} and formaldehyde-C^{14} were incorporated into lignin

in tobacco plant metabolism. The labeled carbon atom of glycine-1-C^{14} was only 2-3% incorporated into the lignin. Serine-3-C^{14} was incorporated into the lignin at the greatest rate followed by formaldehyde-C^{14}, glycine-2-C^{14} and glycine-1-C^{14}.

47. Freudenberg, K., Harkin, J. M., Reichert, M., and Fuzukumi, T., Chem. Ber. 91:581-90(1958).

Studies on various enzyme systems showed that the dehydrogenation reaction of p-hydroxycinnamyl alcohols was nonspecific. Oxygen alone or with copper sulfate gave the same DHP as did the enzymes but at a much slower rate. Aeration in the presence of copper sulfate yielded dehydrodiconiferyl alcohol from coniferyl alcohol, syringaresinol from sinapyl alcohol and dimethoxyquinone from syringaresinol.

48. Freudenberg, K., and Gehrke, G., Chem. Ber. 84:443-51(1951).

Para-coumaryl alcohol, p-coumaraldehyde, their glucosides and DHPs were prepared and compared. The DHP from p-coumaryl alcohol was similar to the DHP from coniferyl alcohol except for the expected low methoxyl content. The ultraviolet spectrum of the DHP from p-coumaryl alcohol was similar to a lignin spectrum. The spectrum of the DHP from p-coumaraldehyde differed greatly from that of lignin.

49. Freudenberg, K., Mitt. d. Osterr. Gesellschaft f. Holzforschung no. 3:72-3(June, 1952).

The theory of lignification for conifers as well as hardwoods was discussed. Furthermore, sinapyl alcohol when mixed with

coniferyl alcohol formed a DHP which was similar to hardwood lignin in all respects. However, sinapyl alcohol alone did not yield a ligninlike polymer.

50. Brown, S. A., and Neish, A. C., Nature 175:688-9(1955).

Randomly labeled shikimic acid, L-phenylalanine and protocatechuic acid were fed to wheat and maple plants. It was found that shikimic acid and L-phenylalanine were efficiently converted into lignin in 24 hrs. When using protocatechuic acid, the lignin was essentially nonradioactive. Other work with labeled benzoic acid, p-hydroxybenzoic acid and anisic acid showed that these aromatic C_6-C_1 compounds were also inefficient as lignin precursors.

51. Eberhardt, G., and Schubert, W. J., J. Am. Chem. Soc. 78:2835-7 (1956).

Shikimic acid, specifically labeled in positions 2 and 6, was incorporated into a living sugar-cane plant. The isolated lignin was radioactive. Nitrobenzene oxidation of this isolated lignin yielded vanillin which was also radioactive but only in positions 2 and 6. It was concluded that this acid was a precursor to the aromatic ring of the lignin building stones.

52. Brown, S. A., and Neish, A. C., Can. J. Biochem. and Physiol. 33:948-62(1955).

Radioactive aromatic monomers which yield phenolic aldehydes on nitrobenzene oxidation were fed to wheat plants. The dilution of radioactivity has been used as a criterion for the monomer's efficiency as a lignin precursor. In general, aromatic C_6-C_1

monomers were not good as lignin precursors, except possibly vanillin and p-hydroxybenzoic acid. Aromatic C_6-C_3 monomers such as tyrosine, phenylalanine, cinnamic acid and ferulic acid were efficient precursors. Ferulic acid was very efficiently converted into the guaiacyl portion only of the lignin.

53. Wright, D., Brown, S. A., and Neish, A. C., Can. J. Biochem. and Physiol. 36:1037-45(1958).

C^{14}-labeled aromatic compounds having side chains of varying length were fed to wheat, buckwheat, sedge, spangletop grass, Salvia splendens, and maple. After 24 hrs., the lignin was isolated and analyzed for radioactivity. p-Hydroxybenzaldehyde was utilized poorly, and mandelic acid was not utilized at all. These facts suggested that the aromatic C_6-C_1 plus C_2 condensation mechanism has limited importance, and that the aromatic C_6-C_2 plus C_1 condensation does not occur at all. Other compounds were tested and found to be efficiently incorporated into lignin. These materials were: DL-phenyllactic acid, L-phenylalanine, DL-phenylhydracrylic acid, and DL-phenylglyceric acid. These findings were consistent with the views that lignin building stones are synthesized through 2-hydroxylated intermediates.

54. Freudenberg, K., and Bittner, F., Chem. Ber. 86:155-9(1953).

Coniferyl alcohol, labeled in the C_1 position of the side chain, was used to prepare DHP and coniferin. The coniferin was fed to a young spruce tree. After two months, the radioactivity had migrated only a few centimeters upward and downward from the point of injection. The bark contained one-third of the radio-

activity. More than 90% of the radioactivity was bound in the lignin. The formaldehyde split from the radioactive DHP was also radioactive, and it was concluded that it originated from the primary carbinol as it occurred in the hydroxymethylcoumaran system of the dehydrodiconiferyl alcohol.

55. Freudenberg, K., and Niedercorn, F., Chem. Ber. 91:591-7(1958).

Phenylalanine, labeled with C^{14} at the beta-carbon atom of the side chain, was incorporated into young spruce stems. After 2-3 days, radioactive coniferin was isolated just above the point of entry. It was concluded that coniferin was an intermediate in lignin biosynthesis.

56. Björkman, A., Svensk Papperstidn. 59:477-85(1956).

Details were presented for the ball milling of finely divided wood. Pre-extracted sprucewood, which had been milled with toluene, yielded by extraction with aqueous dioxane 50% of its original lignin. Evidence was presented which suggested that this lignin preparation was more like protolignin than Braun's isolated native lignin.

57. Siegel, S. M., Physiol. Plant. 6:134-9(1953); C.A. 47:7609.

Thymol and eugenol were converted by embryonic axes of red kidney beans to ligninlike materials. Resorcinol, orcinol, quinol, phloroglucinol and pyrogallol were ineffective. Hydrogen peroxide aided the reaction while KCN served as inhibitor. Peroxidase activity in the plant tissues was localized with the lignin, and

it was suggested that lignification may include the peroxidase-catalyzed oxidation of compounds similar to thymol or eugenol.

58. Siegel, S. M., Physiol. Plant. 7:41-50(1954); C.A. 49:12620.

Eugenol, thymol, ferulic acid and melilotic acid were converted into lignin by sections of Elodea stem internodes. Cinnamic acid and other substituted phenols lacking the C_3 side chain were ineffective. Hydrogen peroxide was also required, and it was deduced that a peroxidase was participating in lignification. Leaves, containing very little peroxidase activity, failed to lignify.

59. Siegel, S. M., Physiol. Plant. 8:20-32(1955); C.A. 50:2761.

The lignin synthesized from a eugenol-hydrogen peroxide mixture by pea-seedling root tips was chemically similar to natural lignin. Color changes during lignification suggested the formation of a semiquinone intermediate. Blocking of the phenolic hydroxyl group inhibited lignification. Phenylacetic acid, tyrosine and pelargonidin chloride were ineffective as lignin precursors. Cyanide, azide, pyrogallol, cysteine, ascorbic acid, and indolacetic acid inhibited lignification.

60. Siegel, S. M., J. Am. Chem. Soc. 78:1753-5(1956).

Model systems of eugenol, enzyme, and peroxide formed water-insoluble oxidation products which failed to yield lignin color tests. However, in the presence of filter paper or methylcellulose, ligninlike materials were formed in the paper or coprecipitated with methylcellulose.

61. Siegel, S. M., J. Am. Chem. Soc. 79:1628-32(1957).

Eugenol-peroxide systems were used in conjunction with macromolecules of various types. Ligninlike materials were synthesized when the macromolecules, regarded as polymerization matrices of low specificity, included celluloses, methylcellulose and chitin. Low yields of ligninlike materials were obtained when proteins and asbestos minerals were used as matrices.

62. Wardrop, A. B., Tappi 40:225-43(1957).

The deposition of lignin in living tissues, based on electron photomicrographs, was discussed. It was shown that lignification began in the primary wall at the cell corners and then extended into the middle lamella and secondary walls. The complementary distribution of peroxidase and lignin suggested that at least one peroxidase-controlled phase of lignification occurred within the cell wall. It was also concluded that, on the basis of this work, lignin precursors originated within individual cells at a particular stage of their differentiation.

63. Mugg, J. B. The methanol-extractable aromatic materials in newly formed aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1958. 92 p.

17 P. tremuloides and 5 P. grandidentata trees were cut throughout the 1956 and 1957 growing seasons. The xylem tissue samples collected represented various stages of lignification. The methanol extracts of these tissues were fractionated according to their solubility in neutral solvents. There were at least ten

different materials which gave positive Mäule and/or Wiesner tests. Free sinapaldehyde was detected in aspen xylem. Mäule- and Wiesner-positive materials were also detected in one phloem extract, but these materials differed from those in the xylem. Evidence was given for the presence in xylem tissue of p-hydroxybenzoic acid, phenylalanine, tyrosine, serine, glycine, and nine other free amino acids. Chromatography of the methanol extracts revealed no difference between aspen trees which could be attributed to sex, physical condition or growth site. No difference could be found between species of P. tremuloides and P. grandidentata.

64. Chang, Y-P. Anatomy of common North American pulpwood barks. TAPPI Monograph Series 14. New York, TAPPI, 1954. 249 p.

Photomicrographs of various sections of P. tremuloides bark and complete details of the bark's anatomy were presented.

65. Sultze, R. F. A study of the phenolic and carbohydrate materials in the newly formed tissues of aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1956. 121 p.

Eleven P. tremuloides trees were felled at various times throughout the growing season. Samples of xylem representing four stages of lignification were collected. These samples were xylem scrapings, soft xylem, new xylem, and year-old xylem. Procedures for collecting these tissues were described. The tissue samples were immediately immersed in methanol to stop enzyme activity. The methanol was changed three times during a 16-day room-temperature extraction. The methanol extracts were concentrated to a water solution. The water solution and precipitated material were further fractionated

into the water-soluble, hexane-soluble and methanol-soluble fractions. Extensive quantitative work on the carbohydrate materials was described.

66. Detrick, R. W. Personal communication. 1958.

Ultraviolet spectral analysis was used for the quantitative determination of sugars which were eluted from paper chromatograms. The paper for chromatography was prepared by a multiple water wash with intermittent air drying. This treatment was sufficient to yield negligible blanks. An acetic acid solution of o-amino-biphenyl was employed as eluting solvent.

67. Bate-Smith, E. C., Chem. & Ind. 1954:1457-8.

Toluene-acetic acid-water (4:1:5) was used successfully for the chromatographic separation of ferulic and caffeic acids found in leaf extracts.

68. Bray, H. G., Lake, H. J., Thorpe, W. V., and White, K., Biochem. J. 47:xiii-xiv(1950).

Benzene saturated with formic acid (98%) was recommended as a developer for phenols such as m-hydroxybenzoic acid and p-hydroxy-benzoic acid.

69. Stone, J., and Blundell, M., Anal. Chem. 23:771-4(1951).

Vanillin and syringaldehyde were separated by paper chromatography with n-butyl ether saturated with water as developer.

70. Wolfe, M., Biochem. et Biophys. Acta 23:186-92(1952).

n-Butanol-acetic acid-water (4:1:5) was used as the first

developer in separating amino acids by two dimensional chromatography.

71. Gailey, W., Chemist Analyst 39:59-62(1950).

n-Butanol saturated with an aqueous solution of 2% ammonia was used to separate vanillin, ethylvanillin and heliotropine by ascending paper chromatography.

72. Fewster, M. E., and Hall, D. A., Nature 168:78-9(1951).

For separating hydroxybenzoic acids, a buffered developer gave more discrete spots. The difficulties in using an acid developer were discussed. The addition of ethanol in some cases enhanced the separation of these acids.

73. Bray, H. G., Thorpe, W. V., and White, K., Biochem. J. 46:271-5 (1950).

Benzene-acetic acid-water (2:2:1) was used to separate monohydroxy acids in rabbit urine.

74. Stanek, D. A. A study of the low-molecular weight phenols formed on the hydrolysis of aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1957. 46 p.

The upper phase of a mixture of n-butanol-pyridine-water (10:3:3) was used to separate vanillic acid, syringic acid and p-hydroxybenzoic acid by paper chromatography. Other Mäule-positive materials isolated from the hot water hydrolyzate of aspenwood were syringaldehyde and sinapaldehyde. It was suggested that p-hydroxybenzoic acid was part of the cell wall of aspenwood.

75. Stark, J. B., Goodban, A. E., and Owens, H. S., Anal. Chem. 23:413-15(1951).

A mixture of 3 g. phenol, 1 ml. water and 1% formic acid was used to separate various organic acids in sugar beet processing liquors.

76. Adler, E., Björkqvist, K. J., and Häggroth, S., Acta Chem. Scand. 2:93-4(1948).

Based on work with the Wiesner reagent using model compounds of the eugenol type and native lignin, it was concluded that the coniferaldehyde group was present in the lignin molecule. The Wiesner reagent was rather specific for p-hydroxycinnamyl aldehydes.

77. Black, R. A., Rosen, A. A., and Adams, S. L., J. Am. Chem. Soc. 75:5344-6(1953).

The Wiesner reagent was shown to give the characteristic red-violet color with sinapaldehyde.

78. Feigl, F. Spot tests. 4th ed. Vol. 2. p. 226. New York, Elsevier Publishing Co., 1954.

A discussion of the phloroglucinol color reaction (Wiesner reagent) was presented. Although there were several exceptions given, the red coloration was probably due to a cinnamyl aldehyde type of configuration. Epihydrin, which was characteristic of the aldehydes present in rancid fats and oils, could be detected by its red color with the Wiesner reagent.

79. Campbell, W. G., McGowan, J. C., and Bryant, S. A., Biochem. J. 32:2138-9(1938).

It was suggested that the so-called Mäule test was specific for the pyrogallol configuration or certain modifications thereof, i.e., methyl ethers.

80. Cross, C. F., Bevan, E. J., and Beadle, C. Cellulose, 2nd ed. p. 115, 194 and 195. New York, Longmans, Green and Co., 1910.

Chlorination of dicotyledonous woods followed by treatment with sodium sulfite gave a brilliant red-violet color.

81. Hough, L., Jones, J. K. N., and Wadman, W., J. Chem. Soc. 1950: 1702-6.

Para-anisidine hydrochloride dissolved in n-butanol was found to be a satisfactory spray reagent for detecting aldohexoses (green-brown), ketohexoses (lemon-yellow), methyl aldepentoses (emerald-green), and uronic acids (cherry-red).

82. Bland, D., Nature 164:1093(1949).

An acid solution of 2,4-dinitrophenylhydrazine was used to identify aldehydes such as vanillin and syringaldehyde on paper chromatograms.

83. Svendson, A. B., Pharm. Acta Helv. 26:253-8(1951); In Block, Durrum and Zweig's Manual of paper chromatography and paper electrophoresis. p. 248. New York, Academic Press Inc., 1955.

Alcoholic ferric chloride was used to detect chlorogenic and caffeic acids on paper chromatograms.

84. Cartwright, R. A., and Roberts, E. A., Chem. and Ind. (London) 1955:230-1.

Quinic acid was detected by the periodate-piperazine-nitroprusside reagent. The chromatogram was first sprayed with a

saturated solution of sodium metaperiodate diluted with two volumes of water, air dried, and then sprayed with a solution of the following composition: sodium nitroprusside, 50 mg.; piperazine, 50 mg.; water, 2 ml.; and absolute ethanol, 10 ml. The chromatogram was then heated at 100°C. for a few minutes to develop the color. Quinic acid gave an orange-yellow spot which sometimes had a green edge.

85. Swain, T., Biochem. J. 53:200-8(1953).

Diazotized p-nitroaniline was used to detect coumarins and related compounds on paper chromatograms. The reagent was prepared as follows: solution A, 0.5% p-nitroaniline in 2N hydrochloric acid; solution B, 5% solution of sodium nitrite; solution C, 20% solution of sodium acetate. Just before using, the spray was prepared by mixing 5 ml. A, 0.5 ml. B, and 15 ml. C in that order. The chromatogram was air dried and oversprayed with a saturated solution of sodium carbonate.

86. Trevelyan, W. E., Procter, D. P., and Harrison, J. S., Nature 166:444-5(1950).

Because of the difficulties encountered in the ammoniacal silver nitrate spray, this reagent was modified in the following way: the dried paper was first passed rapidly through an acetone solution of 3% silver nitrate and allowed to air dry. The paper was then sprayed with a 0.5N solution of sodium hydroxide in aqueous ethanol. Black spots, sugars, appear on a dark background.

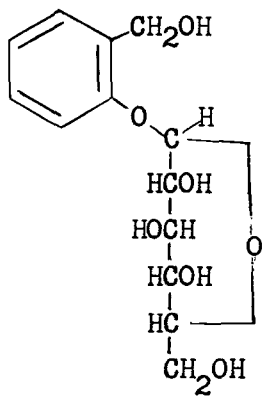
The excess silver oxide (background) was then washed off with 6N ammonium hydroxide followed by a water wash and oven drying.

87. Pearl, I. A. Personal communication, 1958.

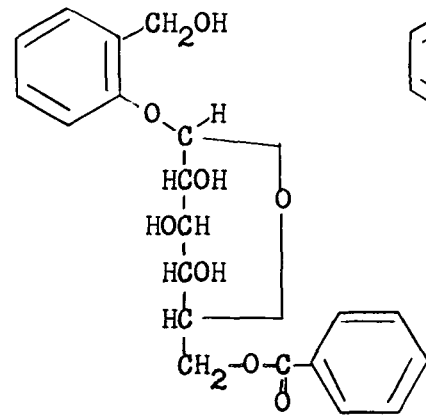
The silver nitrate spray as described by Trevelyan et al. (86) was found to be unsatisfactory for small amounts of glycosidic materials due to background difficulties. Thus, in place of the wash with ammonium hydroxide, Dr. Pearl recommended a wash with a solution of sodium thiosulfate (350 g./l.). After the spots had developed with the ethanolic sodium hydroxide spray, the papers were dipped a few times in sodium thiosulfate and rinsed thoroughly with water. Sugars and glycosidic materials appeared as dense black spots on a nearly white background.

88. Pearl, I. A. Personal communication, 1958.

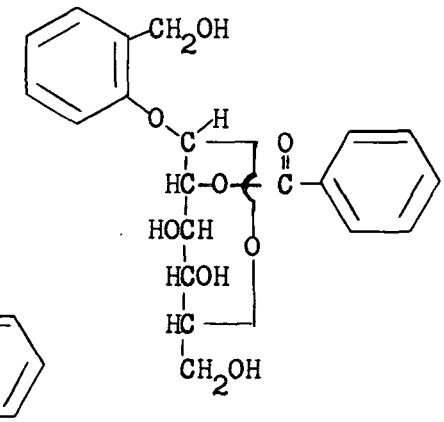
The whole bark of P. tremuloides was extracted with 95% ethanol and analyzed for glycosides. Salicin, populin and a new glucoside, tremuloidin, have been unequivocally identified. The structures of these compounds are shown below.



Salicin



Populin



Tremuloidin

89. Pearl, I. A. Personal communication. 1958.

In his further studies on the glycosides of the whole bark of P. tremuloides, Dr. Pearl has tentatively identified salireposide. Further work leading to the positive identification of this compound is in progress.

90. Birch, A. J. In Todd's Perspectives in organic chemistry. 1st ed. p. 137. New York, Interscience Publishers, Inc., 1956.

The biosynthetic theories in organic chemistry were summarized and discussed in detail.

91. Pearl, I. A., Beyer, D. L., and Johnson, B., Tappi 41:255-6(1958).

Alkaline hydrolysis of 12 hardwoods, cf. (97), revealed the presence of p-hydroxybenzoic acid in the following species: P. nigra, Salix nigra, Salix babylonica and Catalpa speciosa. The only wood outside the Salix family which yielded this acid was Catalpa speciosa.

The wood of P. tremuloides also contained p-coumaric acid in small amounts, but there was no evidence for the presence of ferulic acid.

92. Beyer, D. L. Personal communication. 1958.

Alkaline hydrolyses were performed on the water-soluble fractions of the methanol extracts of aspen whole phloem, soft xylem and year-old xylem prepared by Mugg (63). The following compounds were detected in all samples: p-hydroxybenzoic acid, syringic acid, vanillic acid and p-coumaric acid. It was noted

that in the whole phloem the yield of p-coumaric acid was six to eight times greater than p-hydroxybenzoic acid. In the soft xylem, p-coumaric acid was about four to five times greater than p-hydroxybenzoic acid and in the year-old xylem, p-hydroxybenzoic acid was four to five times greater than p-coumaric acid.

93. Kremers, R. E., Tappi 40:262-8(1957).

A tabulation of the known occurrence of thirteen aromatic compounds which could be considered precursors to lignin was presented.

94. Kremers, R. E., and Reeder, B. J., Personal communication. 1958.

Salicin has been isolated from the soft xylem of P. tremuloides.

95. van Rijn, J. J. L., and Dieterle, H. Die glykoside. Berlin, Gebrüder Borntraeger, 1931. 620 p.

Specific mention was made concerning the reactivities of coniferyl alcohol, syringenin and salicin. These three alcohols were very unstable and condensed in weakly acidic solutions.

96. Freudenberg, K., and Bittner, F., Chem. Ber. 83:600-4(1950).

Details were given for the preparation of coniferyl alcohol from Siam benzoin. Coniferyl alcohol was very sensitive to oxygen, heat, acids and alkalies.

97. Pearl, I. A., Beyer, D. L., Johnson, B., and Wilkinson, S., Tappi 40:374-8(1957).

Alkaline hydrolysis of 34 representative hardwoods revealed the presence of p-hydroxybenzoic acid in the following species only:

Populus tremuloides, P. deltoides, P. trichocarpa var. hastata, P. grandidentata, P. alba, P. tremula, P. tacamahaca and Salix eriocephala.

98. Nakano, J., Ishizu, A., and Migita, N., J. Japan Wood Research Soc. 4, no. 1:1-4(Feb., 1958).

Para-hydroxybenzoic acid was detected in the alkaline hydrolyzates of Larix kampferi, Cryptomeria japonica; Populus maximowiczii, Alnus japonica var. genuina, Fagus crenata, Tilia japonica, Kalopanax pictum var. typicum, Eucalyptus grobulus; Shorea spp. and Octomeles sumatrana. It was concluded that there were ester groups present in the lignin of all the species tested and that the abundance of the ester groups of p-hydroxybenzoic acid was a specific feature of poplar. Evidence was also presented which suggested that the distribution of ester groups in wood was not uniform.

99. Smith, D. C. C., J. Chem. Soc. 1955:2347.

Para-hydroxybenzoic acid was found in "isolated native aspen lignin" by caustic hydrolysis.

100. Busche, L. R. Personal communication. 1959.

Extractive-free aspenwood was subjected to the Klason lignin procedure. The filtrate from this operation contained p-hydroxybenzoic acid. The ethanolic extract of the Klason lignin contained no p-hydroxybenzoic acid. A Klason lignin preparation was then subjected to another Klason procedure, and the filtrate from this operation contained more p-hydroxybenzoic acid. Thus, this acid

could be split off from the lignin during the Klason procedure. It was suggested that some of this acid may have its origin in the lignin.

101. Gonnermann, M., Arch. ges. Physiol. (Pflüger) 123:635-45(1908); C.A. 2:3166.

Pyrocatechol was detected in beets, and it was proposed that it was formed from tyrosine.

102. von Lippmann, E. O., Chem. Ber. 51:272(1918).

Crystalline pyrocatechol was detected on the outer bark surfaces of old sycamore trees.

103. Link, K. P., and Walker, J. C., J. Biol. Chem. 100:379-83(1933).

The resistance to smudge disease by pigmented onions was attributed in part to the presence of pyrocatechol and protocatechuic acid in the outer scales. Similar scales from white onions contained no pyrocatechol.

104. Clagett, C. O., and Tottingham, W. E., J. Agr. Research 62:349-58 (1941); C.A. 35:8137.

The pyrocatechol content of potato tubers correlated with the degree of blackening upon boiling.

105. Dupuy, P., and Puisais, J., Compt. rend. 241:48-51(1955); C.A. 49:16076.

Pyrocatechol was chromatographically detected in the leaves and fruit of grape vines.

106. Kritchevsky, G., and Anderson, A. B., J. Org. Chem. 20:1402-6(1955).

Pyrocatechol was chromatographically detected among the degredation products, i.e., acid hydrolysis and alkali fusion, of the cone solids from Sequoia sempervirens and Sequoia gigantea. Reference was also made to the fact that Sequoia forests were quite resistant to fungi and insect attacks.

107. Karrer, P., and Matter, E., Helv. Chim. Acta 29:1871-2(1946).

Pyrocatechol was found in Calabash curare.

108. Nair, P. V., and Punnoose, T. V., Bull. Central Research Inst., Univ. Travancore, Ser. A, 1, no. 1:87-102(1950); C.A. 46:11675.

Pyrocatechol was detected in Odier wodier bark.

109. Plakhova, N. B., Farmakol. i Toksikol. 17, no. 4:39-42(1954); C.A. 48:13820.

Burnet (Sanguisorba officinales), tormentil, bistort, Bergenia crassifolia, horse sorrel (Rumex confertus), and Finnish sorrel (Rumex fenicus) yielded antiseptic tannins mainly from the roots. These tannin extracts contained pyrocatechol, pyrogallol, resorcinol, hydroquinone and gallic acid.

110. Isenberg, I. H. Pulpwoods of the United States and Canada. 2d ed. p. 94. Appleton, Wis., The Institute of Paper Chemistry, 1951.

P. tremuloides exhibited low resistance to several decay micro-organisms.

111. Terui, G., Enatsu, T., and Tokaku, H., J. Fermentation Technol. (Japan) 31:65-71(1953); C.A. 47:8113.

A strain of A. niger decomposed salicylic acid yielding

pyrocatechol, oxalic acid and carbon dioxide. 2,3-Dihydroxybenzoic acid was an intermediate to both pyrocatechol and oxalic acid. Pyrocatechol did not give rise to oxalic acid.

112. Hughes, H. K., Anal. Chem. 24:1349-54(1952).

The accepted nomenclature for use in applied spectroscopy was presented.

113. Aulin-Erdtman, G., Svensk Kem. Tidskr. 70:145-56(1958).

The $\Delta\epsilon$ method for quantitative and qualitative analysis of an ultraviolet spectrum was described. The author proposed a modification of the earlier work by using model compound studies to correct various ultraviolet spectra of mixtures. Thus, a new curve could be obtained which had been "reduced" by known factors, and this reduced curve could then be analyzed again by the same $\Delta\epsilon$ method. Mention was also made of the possibility of applying this technique to the infrared region.

APPENDIX

INFRARED SPECTRAL ANALYSIS OF THE VARIOUS FRACTIONS

During the standard fractionation procedure involving T-81,82 inner phloem and stone cell layer, the materials used for solids determinations were also used for infrared spectral analysis. It was thought that such an analysis might prove useful both to determine qualitatively whether a given solvent fractionation was successful and to aid in subsequent analysis of each fraction. It was hoped that chemical groupings such as aliphatic, carbonyl etc. could be put on a relative semiquantitative basis. Thus, eighteen spectra were obtained representing the various fractions of the two tissue extracts.

A qualitative examination of the curves, shown in Figures 16, 17, 18 and 19, did not reveal any apparent clean-cut fractionation. However, if Beer's Law is assumed, a fairly good qualitative methematical treatment of these data can be obtained.

Beer's Law describes the absorption in a beam of parallel, monochromatic radiation in a homogeneous, isotropic medium (112).

$$\underline{P} = \underline{P}_o 10^{-\underline{abc}} \qquad (1)$$

where:

$\underline{P}$ = transmitted radiant power
$\underline{P}_o$ = incident radiant power, or a quantity proportional to it as measured with a pure solvent in the beam
$\underline{a}$ = absorptivity, a constant characteristic of the material and the frequency
$\underline{b}$ = internal cell length in cm., assumed constant
$\underline{c}$ = concentration
$\underline{A} = \underline{abc}$ = absorbance

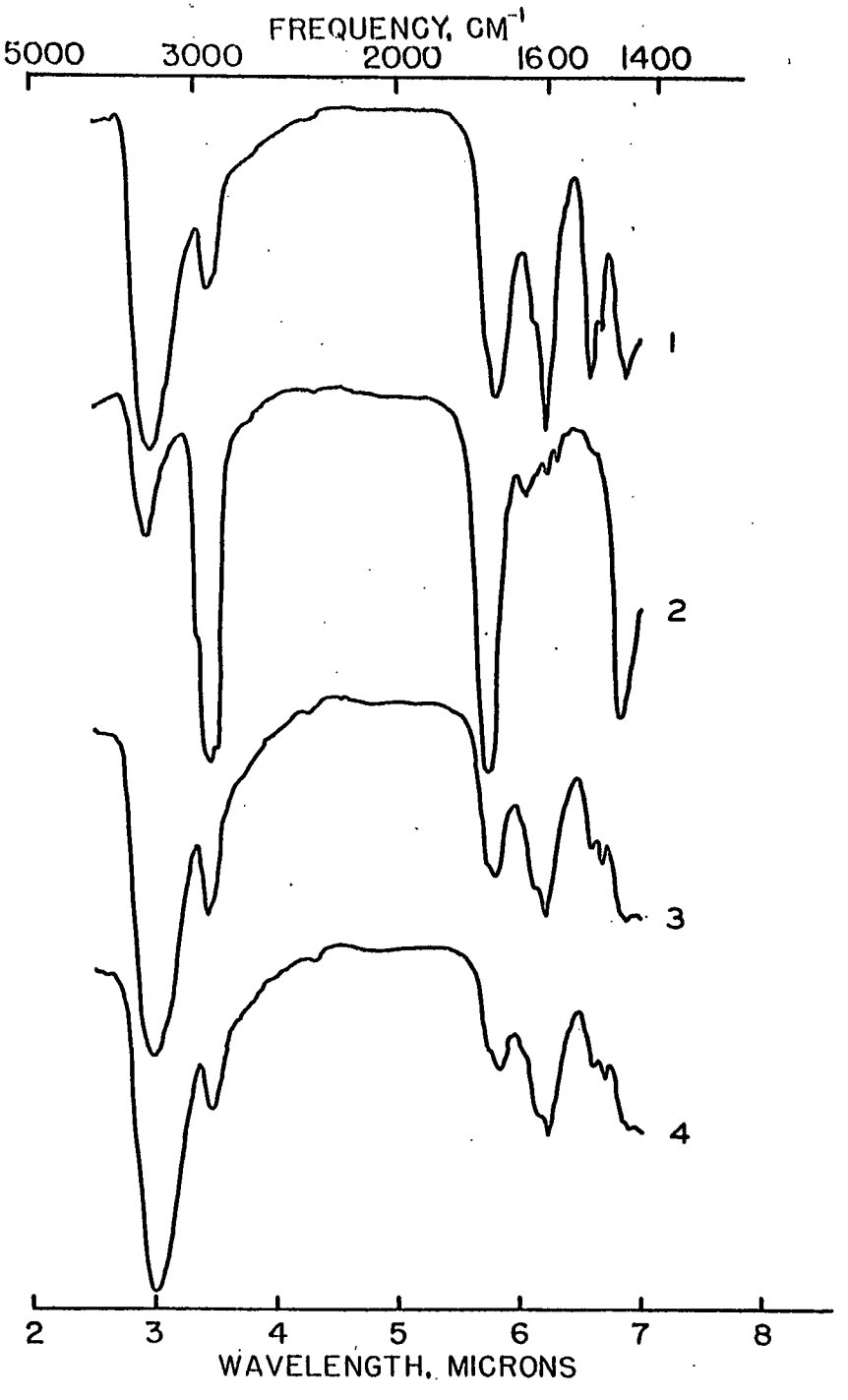


Figure 16. Infrared Spectra of the Precipitate-from-water (1), the Hexane-soluble Materials (2), the Water-soluble Materials (3), and the Water-soluble, Ether-insoluble Materials (4). T-81,82 Inner Phloem

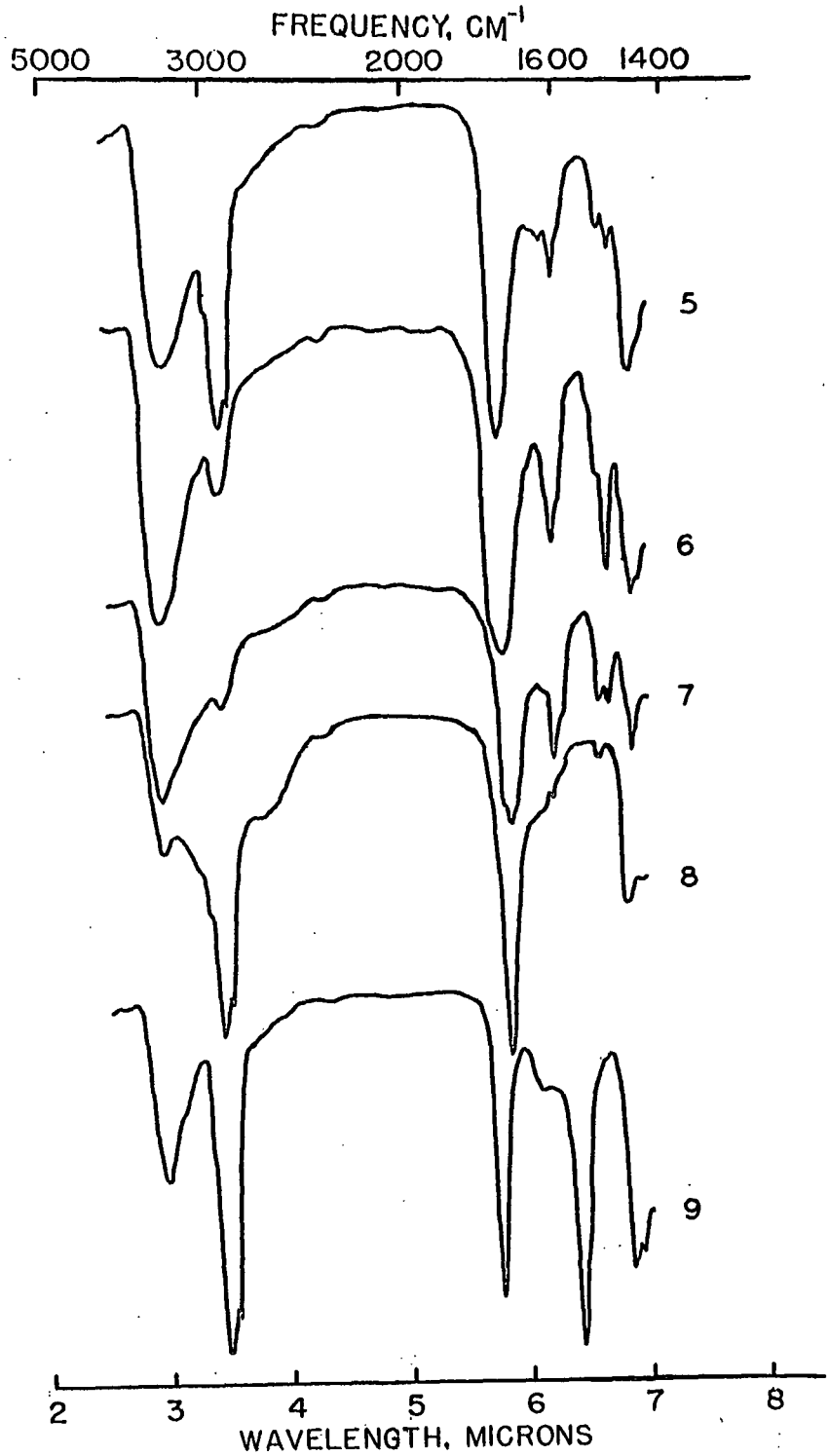


Figure 17. Infrared Spectra of the Precipitate-from-ether (5), the Bisulfite-soluble Materials (6), the Bicarbonate-soluble Materials (7), the Sodium Hydroxide-soluble Materials (8), and the Ether Neutrals (9). T-81,82 Inner Phloem

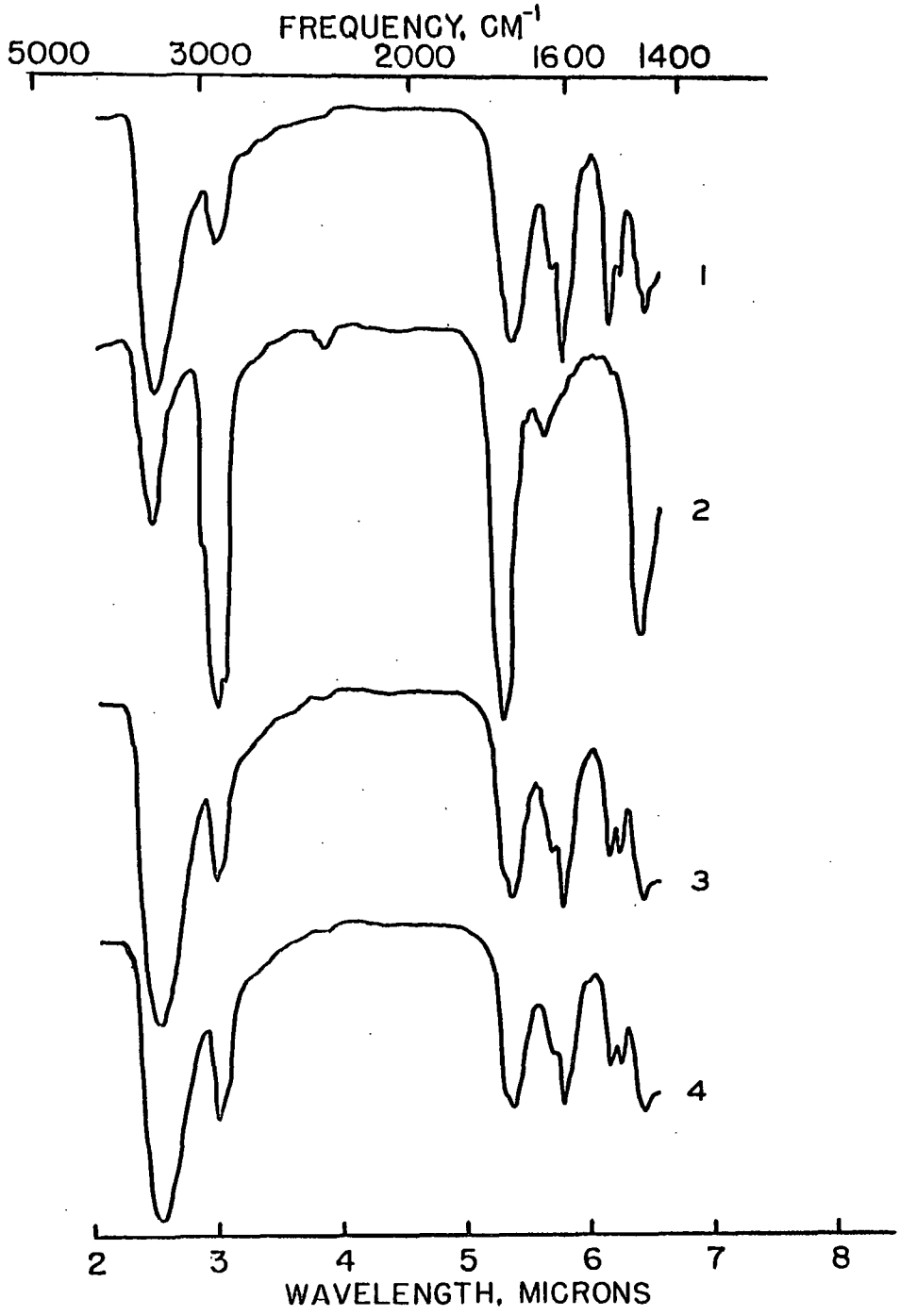


Figure 18. Infrared Spectra of the Precipitate-from-water (1), the Hexane-soluble Materials (2), the Water-soluble Materials (3), and the Water-soluble, Ether-insoluble Materials (4). T-81,82 Stone Cell Layer

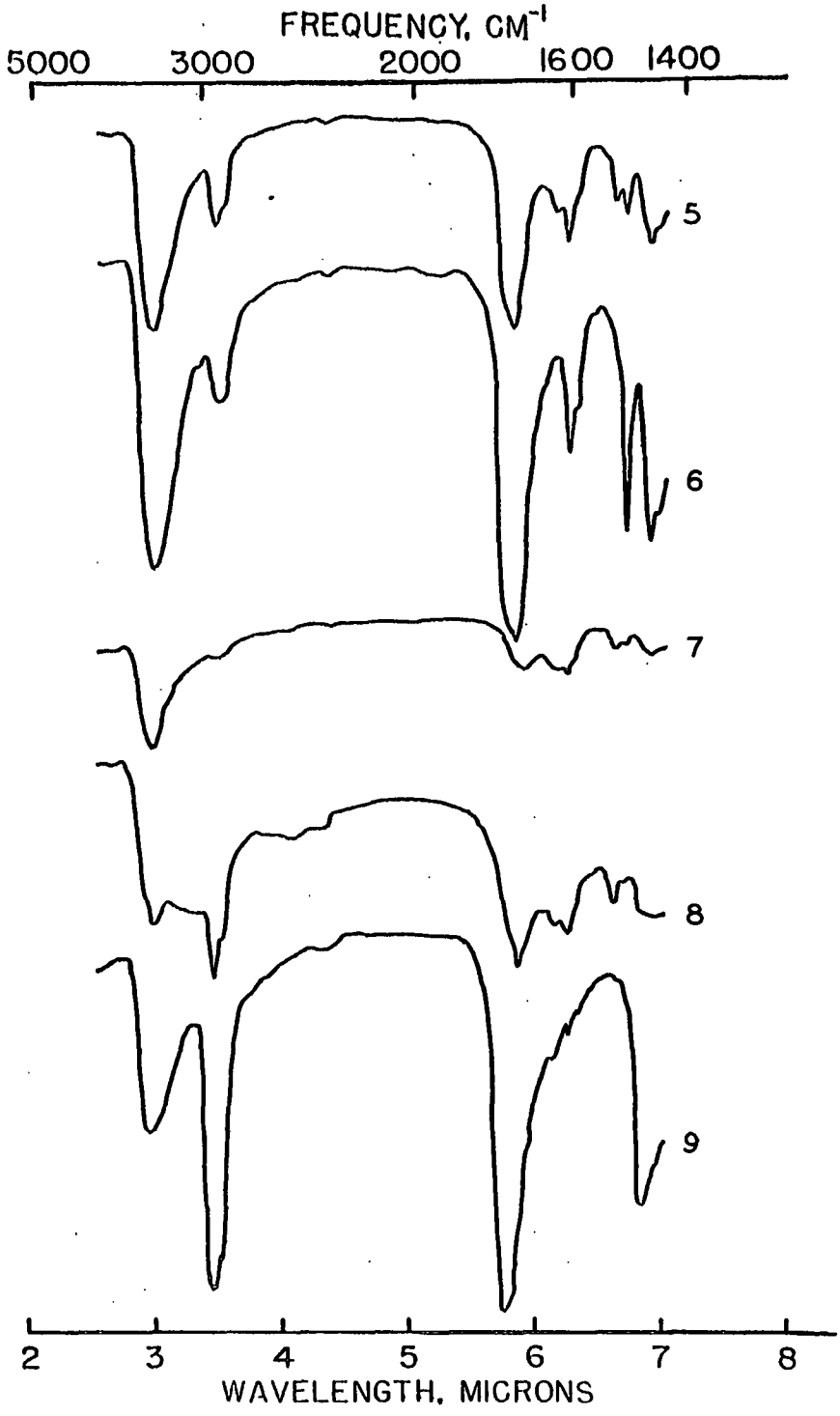


Figure 19. Infrared Spectra of the Precipitate-from-ether (5), the Bisulfite-soluble Materials (6), the Bicarbonate-soluble Materials (7), the Sodium Hydroxide-soluble Materials (8), and the Ether Neutrals (9). T-81,82 Stone Cell Layer

For a given material, containing functional groups 1 and 2, the ratio of the absorbances of these two functional groups is proportional to the relative concentrations of these groups; thus, rearranging Equation (1), one obtains a measure of the relative amounts of each

$$\frac{A_1}{A_2} = \frac{-\log\frac{P_1}{P_0}}{-\log\frac{P_2}{P_0}} = \frac{K_1 c_1}{K_2 c_2} = \frac{c_1}{c_2} \times K_{1,2} \qquad (2)$$

where:

$K_1 = a_1 b$ = constant

$K_2 = a_2 b$ = constant

and $K_{1,2} = K_1/K_2$ = constant

functional group considered.

The infrared spectrum of a given material usually plots % transmission vs. frequency. The functional groups considered in this analysis were the hydroxyl, aliphatic, carbonyl, and aromatic, and whose absorption maxima occur at 3340-3400 $cm.^{-1}$, 2880-2920 $cm.^{-1}$, 1705-1745 $cm.^{-1}$, and 1560-1650 $cm.^{-1}$, respectively. For a given fraction, the values of P for each functional group can be read from the infrared curve. The value of P_0 was taken as the average value of P between the frequencies 1900-2200 $cm.^{-1}$. The values of P read from the infrared curve are per cent values and not absolute. However, since only ratios are desired, these values are satisfactory. These data appear in Tables XVIII and XIX.

TABLE XVIII

PER CENT TRANSMISSION VALUES FOR VARIOUS CHEMICAL GROUPINGS

T-81,82 Inner Phloem

Fraction	Group P_h [a]	P_{al}	P_c	P_{ar}	P_o
1. Precipitate-from-water	14	47	25	18	84
2. Hexane-soluble materials	51	4	2	60	81
3. Water solution, hexane ext'd.	8	37	45	36	81
4. Water solution, hexane and ether ext'd.	12	50	58	43	83
5. Precipitate-from-ether	24	11	8	42	78
6. HSO_3^--soluble materials	15	42	8	31	77
7. HCO_3^--soluble materials	37	56	32	46	82
8. NaOH-soluble materials	49	10	6	61	78
9. Ether neutrals	44	7	18	7	82

[a] P_h --- $P_{hydroxyl}$
P_{al} --- $P_{aliphatic}$
P_c --- $P_{carbonyl}$
P_{ar} --- $P_{aromatic}$

TABLE XIX

PER CENT TRANSMISSION VALUES FOR VARIOUS CHEMICAL GROUPINGS

T-81,82 Stone Cell Layer

Fraction	Group P_h [a]	P_{al}	P_c	P_{ar}	P_o
1. Precipitate-from-water	29	60	40	36	87
2. Hexane-soluble materials	43	5	2	60	83
3. Water solution, hexane ext'd.	16	45	41	40	84
4. Water solution, hexane and ether ext'd.	25	45	48	48	83
5. Precipitate-from-ether	41	62	42	59	86
6. HSO_3^--soluble materials	24	58	10	48	84
7. HCO_3^--soluble materials	57	75	74	73	84
8. NaOH-soluble materials	43	32	34	42	72
9. Ether neutrals	42	10	6	63	82

[a] P_h --- $P_{hydroxyl}$
P_{al} --- $P_{aliphatic}$
P_c --- $P_{carbonyl}$
P_{ar} --- $P_{aromatic}$

Using Equation (2), the ratios of the concentrations of the various functional groups may be calculated. The results of these calculations appear in Tables XX and XXI. The aliphatic functional group concentration was used as a reference. It should be noted that each column represents a relative concentration times a constant. This constant is different for each of the three columns.

While these ratios do not represent absolute values, they do indicate a qualitative trend and are generally consistent within each column of ratios. As an example, refer to Table XX, column 2. Carbonyl groups would be expected to appear predominantly in the bisulfite, hexane, precipitate-from-water, and bicarbonate fractions. These four fractions have relatively high carbonyl/aliphatic ratios, thus supporting the original expectation. One would also expect fatty materials to predominate in the hexane fraction, and the ratio of hydroxyl/aliphatic to be quite small. This expectation is borne out in Tables XX and XXI, Fraction 2.

This type of spectral analysis may be useful in analyzing such materials as isolated lignins or high polymer mixtures in general. Aulin-Erdtman (113) has described a mathematical method for treating the ultraviolet spectrum of a mixture of compounds. This method was quite useful when one or more components of the mixture were known, and it was suggested that this method might be extended into the infrared region. The method described herein is in agreement with the suggestion of Aulin-Erdtman.

TABLE XX

RELATIVE AMOUNTS OF VARIOUS CHEMICAL GROUPS

T-81,82 Inner Phloem

Fraction	Ratio $\underline{c}_h/\underline{c}_{al} \times \underline{K}_1$ [a]	$\underline{c}_c/\underline{c}_{al} \times \underline{K}_2$	$\underline{c}_{ar}/\underline{c}_{al} \times \underline{K}_3$
1. Precipitate-from-water	3.2	2.1	2.7
2. Hexane-soluble materials	0.2	1.3	0.1
3. Water solution, hexane ext'd.	3.0	0.8	1.0
4. Water solution, hexane and ether ext'd.	3.8	0.7	1.3
5. Precipitate-from-ether	0.6	1.2	0.3
6. HSO_3^--soluble materials	2.7	3.7	1.5
7. HCO_3^--soluble materials	2.1	2.5	1.5
8. NaOH-soluble materials	0.2	1.3	0.1
9. Ether neutrals	0.3	0.6	1.0

[a] $\underline{c}_h$ --- $\underline{c}_{hydroxyl}$
$\underline{c}_{al}$ --- $\underline{c}_{aliphatic}$
$\underline{c}_c$ --- $\underline{c}_{carbonyl}$
$\underline{c}_{ar}$ --- $\underline{c}_{aromatic}$

$\underline{K}_1 - \underline{K}_{hydroxyl}/\underline{K}_{aliphatic}$
$\underline{K}_2 - \underline{K}_{carbonyl}/\underline{K}_{aliphatic}$
$\underline{K}_3 - \underline{K}_{aromatic}/\underline{K}_{aliphatic}$

TABLE XXI

RELATIVE AMOUNTS OF VARIOUS CHEMICAL GROUPS

T-81,82 Stone Cell Layer

Fraction	Ratio $\underline{c}_h/\underline{c}_{al} \times \underline{K}_1$ [a]	$\underline{c}_c/\underline{c}_{al} \times \underline{K}_2$	$\underline{c}_{ar}/\underline{c}_{al} \times \underline{K}_3$
1. Precipitate-from-water	2.9	2.1	2.4
2. Hexane-soluble materials	0.2	1.3	0.1
3. Water solution, hexane ext'd.	2.7	1.2	1.2
4. Water solution, hexane and ether ext'd.	1.9	0.9	0.9
5. Precipitate-from-ether	2.4	2.3	1.2
6. HSO_3^--soluble materials	3.4	5.7	1.5
7. HCO_3^--soluble materials	3.5	1.1	1.2
8. NaOH-soluble materials	0.6	0.9	0.7
9. Ether neutrals	0.3	1.2	0.1

[a] $\underline{c}_h$ --- $\underline{c}_{hydroxyl}$
$\underline{c}_{al}$ --- $\underline{c}_{aliphatic}$
$\underline{c}_c$ --- $\underline{c}_{carbonyl}$
$\underline{c}_{ar}$ --- $\underline{c}_{aromatic}$

$\underline{K}_1$ -- $\underline{K}_{hydroxyl}/\underline{K}_{aliphatic}$
$\underline{K}_2$ -- $\underline{K}_{carbonyl}/\underline{K}_{aliphatic}$
$\underline{K}_3$ -- $\underline{K}_{aromatic}/\underline{K}_{aliphatic}$